UNIVERSIDADE DE LISBOA
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Endolysins as Antibacterial Agents: from Engineering Approaches to the Uncovering of Holin as a Key Factor Influencing Lytic Activity

Orientador: Prof. Doutor Carlos Jorge Sousa de São-José

Ana Sofia Conceição Fernandes

Tese especialmente elaborada para a obtenção do grau de doutor em Farmácia, especialidade de Microbiologia.

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RESUMO

Os bacteriófagos, ou fagos, são vírus que infetam exclusivamente bactérias. Os fagos de DNA de cadeia dupla utilizam o sistema holina-endolisina para lisar a célula hospedeira, garantindo deste modo a libertação da descendência viral e a realização de novos ciclos de infeção. A endolisina é uma enzima que degrada o peptidoglicano (PG), o principal constituinte da parede celular (CW) bacteriana. A holina é uma proteína transmembranar que forma poros na membrana citoplasmática (CM), os quais levam à dissipação da força proto motriz (pmf) e consequentemente à morte celular. A pmf deriva do gradiente eletroquímico formado através da CM, sendo essencial para a realização de funções que requerem energia celular. Considerando o modo como as endolisinas acedem à CW, estas podem classificar-se em dois tipos: i) endolisinas canónicas (c-endolisinas), quando o acesso é feito através dos poros das holinas, ou ii) endolisinas exportadas (e-endolisinas), quando o transporte ocorre por uma via independente da holina. Considera-se que, uma vez sintetizadas, as c-endolisinas adquirem de imediato a sua conformação ativa no citoplasma, tendo portanto a capacidade de degradar eficientemente o PG se o contacto com a CW for permitido. Embora exportadas para a CW pela maquinaria da célula hospedeira, as e-endolisinas descritas até à data necessitam de ser ativadas por mecanismos que dependem da ação da holina.

Devido à sua atividade lítica, as c-endolisinas têm ganho grande destaque como potenciais agentes antimicrobianos, para a eliminação de bactérias patogénicas Gram-positivas, principalmente no contexto do crescente aumento das resistências aos antibióticos. Esta abordagem assenta na observação de que, pelo menos em certas condições, as c-endolisinas são capazes de lisar de forma eficiente bactérias alvo, quando as enzimas são adicionadas exogenamente, na forma de proteínas recombinantes (enzibióticos). A realização deste trabalho teve como objetivo principal o de contribuir com conhecimentos que permitam melhorar o potencial das endolisinas enquanto agentes antibacterianos, quer pelo desenvolvimento de estratégias visando o aumento da sua produção, solubilidade e ação lítica, quer pela compreensão de fatores e mecanismos que influenciam a sua atividade.

Este trabalho iniciou-se com a construção de endolisinas quiméricas com ação lítica sobre *Staphylococcus aureus* (Capítulo 2). Para além da obtenção de enzimas com largo espectro de ação sobre estirpes clínicas desta espécie, pretendia-se também obviar o problema frequente da baixa solubilidade das endolisinas dos fagos de *S. aureus*, quando estas são superproduzidas em *Escherichia coli*. Produziram-se e purificaram-se duas proteínas quiméricas (Lys168-87 e Lys170-87), em que o mesmo domínio de ligação à parede celular (CWBD) da endolisina Lys87, produzida pelo fago de *S. aureus* F87s/06, foi fundido ao domínio catalítico (CD) das endolisinas Lys168 ou Lys170, produzidas pelos fagos de *Enterococcus faecalis* F168/08 e F170/08, respetivamente. A fusão entre o CD de endolisinas solúveis e o CWBD de uma endolisina com fraca solubilidade, aliada a condições de expressão otimizadas, permitiu que se produzissem eficazmente enzimas quiméricas na forma solúvel. A atividade lítica das quimeras foi inicialmente avaliada qualitativamente por "spot assay" em isolados bacterianos obtidos em meio hospitalar e na comunidade Portuguesa (n = 100). As endolisinas evidenciaram um elevado potencial lítico, apresentando atividade em mais de 90% dos isolados de *S. aureus* testados, incluindo uma elevada fração destes com resistência à meticilina (MRSA; n = 42). Esta capacidade lítica foi igualmente observada numa coleção de estirpes de *S. aureus* geneticamente caracterizadas, a qual abrangia representantes dos clones pandémicos MRSA mais relevantes, isolados em diferentes partes do mundo (n= 30), e clones dominantes de *S. aureus* sensíveis à meticilina (MSSA; n= 13). Em ensaios semiquantitativos (curvas de lise), as endolisinas Lys168-87 e Lys170-87 foram eficazes na eliminação da estirpe MRSA USA200, quando testadas num tampão fisiológico, registando-se um efeito sinergístico pela ação conjunta das duas quimeras. Curiosamente, ao contrário das endolisinas parentais, as quimeras apresentaram um espectro de ação alargado, sendo também ativas contra *Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus saprophyticus, E. faecalis, Enterococcus faecium* e *Streptococcus pyogenes*. Globalmente, Lys168-87 apresentou uma atividade lítica superior à de Lys170-87, invertendo-se esta característica apenas quando testadas em *Enterococcus* spp.

Os resultados da nossa investigação e de muitos outros laboratórios suportam a capacidade lítica das c-endolisinas e derivados quiméricos quando testados *in vitro*, nomeadamente quando as células alvo são transferidas para tampões fisiológicos antes da adição das enzimas. No entanto, verificámos que essa eficácia lítica é frequentemente

perdida ou significativamente diminuída quando as bactérias alvo são mantidas em meios nutritivos, i.e., em condições que garantem a manutenção da pmf e um crescimento celular ativo. Assim, na segunda parte do trabalho pretendeu-se elucidar que fatores e mecanismos poderiam estar na base deste fenómeno. Nesse sentido, fomos guiados pelo facto de que no contexto natural da infeção fágica ambos os tipos de endolisinas, c- e e-endolisinas, atuam sempre em células hospedeiras previamente mortas pela ação da holina. Tendo por base esta observação, decidiu-se estudar o modo como a manutenção ou a dissipação da pmf poderia influenciar a atividade das c-endolisinas, quer quando estas alcançam a CW pelo interior da célula, quer quando são aplicadas externamente. Selecionou-se como exemplo de c-endolisina a do fago SPP1 de *Bacillus subtilis* (LysSPP1) e procedeu-se inicialmente à sua transformação numa e-endolisina artificial. Para isso, fundiu-se a sequência sinal (SP) da bacilopeptidase F de *B. subtilis* (proteína Bpr) com a extremidade N-terminal de LysSPP1. O gene recombinante da e-endolisina artificial SP-LysSPP1 foi clonado num plasmídeo replicativo em *B. subtilis* sob o controlo de um promotor induzível. Surpreendentemente, a produção e exportação continuada de SP-LysSPP1 para a CW, através do sistema de secreção (sistema Sec) de *B. subtilis*, não produziu efeitos óbvios ao nível da viabilidade e crescimento celular, quando comparados com o comportamento de uma estirpe produtora da versão nativa, não exportada, de LysSPP1. A localização extracitoplasmática e o carácter lítico da forma madura de SP-LysSPP1 foram evidenciados após o tratamento das culturas com um ionóforo dissipador da pmf, a gramicidina D. Tal como esperado, a adição deste ionóforo resultou na estagnação do crescimento das culturas em estudo. No entanto, apenas a estirpe produtora de SP-LysSPP1 apresentou lise celular imediata após a adição de gramicidina D. Paralelamente, demonstrou-se que a sensibilização prévia de *B. subtilis* com gramicidina D ou a sua manutenção em meio tamponado sem fontes de energia, potencia significativamente a atividade lítica da endolisina quando adicionada exogenamente. Estimou-se que a quantidade de LysSPP1 necessária para lisar células previamente mortas pela ação da holina de SPP1, ou pela ação de um agente dissipador da pmf, é cerca de 60 vezes menor do que a quantidade necessária para lisar células em crescimento exponencial ativo. A mesma linha de resultados foi obtida quando se testou a atividade lítica da c-endolisina Lys11, do fago ϕ11 de *S. aureus*, em condições promotoras ou inibidoras da pmf/crescimento. Os resultados obtidos demonstram assim que a dissipação da pmf, que no contexto da infeção fágica ocorre pela ação da holina, pode ter um efeito ativador ou potenciador da ação lítica das c-endolisinas, tal como descrito anteriormente

para as e-endolisinas. Curiosamente, esta característica tem sido igualmente observada em outras hidrólases do PG, como as autolisinas, cuja regulação está dependente da manutenção da pmf. Estes resultados podem ter implicações na seleção e desenho de endolisinas destinadas à enzibioterapia (ver Capítulo 3).

Finalmente, uma terceira parte do trabalho teve por objetivo identificar e caracterizar a função de holina do fago SPP1, condição necessária para conduzir parte dos estudos apresentados no Capítulo 3. Foi anteriormente proposto que a holina de SPP1 seria codificada pela *orf 26*. O seu produto (gp26) partilha homologia e características hidrofóbicas com a proteína XhlB, a qual foi implicada na função holina do fago críptico PBSX de *B. subtilis*. Contudo, a nossa análise revelou a montante a *orf 24.1* que codifica para uma proteína do tipo holina análoga à XhlA, também envolvida na lise medida pelo PBSX. Assim, decidiu-se estudar o papel de gp24.1 e gp26 enquanto possíveis holinas do fago SPP1. Dada a sua potencial toxicidade, frequentemente as holinas de sistemas Gram-positivos são estudadas em sistemas heterólogos, tipicamente em *E. coli*. Contudo, ainda que apresentando algumas vantagens, por vezes torna-se difícil a extrapolação dos resultados para os sistemas nativos. Deste modo, para se perceber o contributo de gp24.1 e gp26 para a função de holina do fago SPP1, as respetivas *orfs* foram clonadas em separado e na forma de uma fusão transcricional num plasmídeo replicativo em *B. subtilis* e sob a dependência de um promotor induzível. Os resultados revelaram que, nas condições de expressão utilizadas, a produção individual destas proteínas não tem impacto significativo no crescimento celular de *B. subtilis*, apesar de se inserirem na CM. A cessação do crescimento e morte celular típicas da ação da holina só foram observadas após coprodução de gp24.1 e gp26, sugerindo que em SPP1 a função holina poderá envolver a produção destas duas proteínas. Surpreendentemente, foi identificado um promotor constitutivo interno à *orf 24.1*, o qual deverá corresponder ao promotor precoce *PE5* anteriormente descrito para o fago SPP1. A presença deste promotor levanta questões relativamente à regulação da lise neste fago (ver Capítulo 4).

Palavras-chave: endolisina, holina, potencial de membrana, pmf, *B. subtilis*, SPP1, *S. aureus*, endolisina quimérica, resistência aos antibióticos.

ABSTRACT

Bacteriophages, or phages, are viruses that strictly infect bacteria. Double-stranded DNA phages use the holin-endolysin system to lyse host cells, thus ensuring the release of the viral progeny and the realization of new infection cycles. The endolysin is an enzyme that degrades the peptidoglycan (PG), the main constituent of the bacteria cell wall (CW). The holin is a transmembrane protein that forms pores in the cytoplasmic membrane (CM), leading to dissipation of the proton motive force (pmf) and consequently to the cell death. The pmf is created by the electrochemical gradient across the CM and is responsible for driving many energy-requiring functions in the cell. According to the way how endolysins reach the CW, these can be classified into two types: i) canonical endolysins (c-endolysins), when access occurs through the holin pores, or ii) exported endolysins (e-endolysins), when transport is performed in a holin-independent pathway. It is considered that once synthesized c-endolysins immediately acquire their active conformation in the cytoplasm, thus having the capacity to effectively degrade the PG if the contact with the CW is allowed. Although exported to the CW by the host cell machinery, all e-endolysins described so far need to be activated by mechanisms that depend on the holin action.

Due to their lytic activity, c-endolysins have gained great attention as potential antimicrobial agents for the elimination of pathogenic Gram-positive bacteria, especially in the actual context of increasing resistance to antibiotics. This approach relies on the observation that, at least under certain conditions, c-endolysins are able to efficiently lyse target bacteria when the enzymes are exogenously added in the form of recombinant proteins (enzybiotics). The main objective of the work here presented was to contribute with knowledge for increasing the potential of endolysins as antibacterial agents, namely by developing strategies to improve their production, solubility and lytic performance, and by deepening our understanding of factors and mechanisms that influence their enzymatic activity.

This work began with the construction of chimerical endolysins with lytic activity against *Staphylococcus aureus* (Chapter 2). In addition to obtain enzymes displaying a broad lytic spectrum on clinical strains of this species, we also intended to overcome the problem of low solubility that is commonly observed when overproducing endolysins of *S. aureus* phages in *Escherichia coli*. We produced and purified two chimerical proteins (Lys168-

87 and Lys170-87) by fusing the same cell wall binding domain (CWBD) of endolysin Lys87, produced by *S. aureus* phage F87s/06, to the catalytic domain (CD) of endolysins Lys168 or Lys170, produced by *Enterococcus faecalis* phages F168/08 and F170/08, respectively. This fusion between the CD of highly soluble endolysins and the CWBD of an endolysin with poor solubility, combined with optimized expression conditions, allowed the efficient production of chimerical enzymes in the soluble form. The lytic activity of the chimeras was initially assessed qualitatively by the "spot assay", using bacterial isolates from Portuguese community and hospital settings (n = 100). The endolysins showed high lytic potential, lysing more than 90% of the tested *S. aureus* isolates, including a high fraction that was methicillin-resistant (MRSA, n = 42). This lytic capacity was also observed in a collection of genetically characterized and typed *S. aureus* strains, which included representatives of the most relevant MRSA pandemic clones from different parts of the world (n = 30), and representative clones of the dominant methicillin-sensitive *S. aureus* (MSSA; n = 13). In semi-quantitative assays (lysis curves), Lys168-87 and Lys170-87 were effective in eliminating MRSA strain USA200 suspended in a physiological buffer, being observed a synergistic effect when the chimeras were simultaneously used. Interestingly, unlike the parental endolysins, the chimeras showed a wide lytic spectrum, being also active against *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus*, *E. faecalis*, *Enterococcus faecium* and *Streptococcus pyogenes*. Globally, Lys168-87 presented superior lytic performance than Lys170-87, being this result only inverted when the chimeras were tested on the enterococcal isolates.

The results of our research and from many other laboratories support the lytic capacity of c-endolysins and chimeric derivatives when tested *in vitro*, particularly when the enzymes are added to cells previously suspended in physiological buffers. Nevertheless, we have found that lytic efficacy is often lost or greatly lessened when target bacteria are kept in nutritious media, i.e., in conditions that guarantee maintenance of the pmf and active cell growth. Thus, in a second part of this work we aimed at uncovering factors and mechanisms that underlie this phenomenon. Accordingly, we were led by the fact that in the phage infection context both c- and e-endolysins always act after cells have been killed by the holin. Based on this observation, we decided to study how maintenance or dissipation of the pmf could influence the activity of c-endolysins, either when they reach the CW from the cell inside or when applied externally. The c-endolysin of *Bacillus*

subtilis phage SPP1 (LysSPP1) was chosen as study model, being in a first step transformed into an artificial e-endolysin. For this, the signal sequence (SP) of bacillopeptidase F of *B. subtilis* (Bpr protein) was fused to the N-terminal end of LysSPP1. The recombinant gene of the artificial e-endolysin SP-LysSPP1 was cloned in a *B. subtilis* replicative plasmid under the control of an inducible promoter. Surprisingly, the continued production and export of SP-LysSPP1 to the CW, through the secretion system (Sec system) of *B. subtilis*, produced no obvious effects on the viability and cell growth when compared with a strain that produced the native and not exported LysSPP1. The extracytoplasmic localization and lytic character of the mature form of SP-LysSPP1 was evidenced when cultures were treated with a pmf-dissipating ionophore, gramicidin D. As expected, the addition of the ionophore resulted in immediate growth interruption. However, only the culture producing SP-LysSPP1 showed quick cell lysis after addition of gramicidin D. Similarly, the prior sensitization of *B. subtilis* with gramicidin D or the maintenance of cells in a buffered medium without energy sources significantly enhanced the lytic activity of the endolysin when added exogenously. It was estimated that the amount of LysSPP1 necessary to lyse cells previously killed by the SPP1 holin action, or by a pmf-dissipating agent, was about 60 times lower than the amount needed to lyse exponentially growing cells. The results followed the same trend when the c-endolysin Lys11, of *S. aureus* phage ϕ11, was tested in conditions promoting or decreasing the pmf/cell growth. Therefore, the results demonstrate that dissipation of the pmf, which in the context of phage infection occurs by the holin action, can have an activating or potentiating effect on the lytic action of c-endolysins, similarly to what was previously described for e-endolysins. Interestingly, this feature has also been observed with other PG hydrolases, such as autolysins, whose regulation is dependent on the maintenance of the pmf. These results may have implications on the selection and design of endolysins intended for enzybiotic therapy (see Chapter 3).

Finally, in a third part of this work we aimed to identify and characterize the holin function of phage SPP1, a necessary condition to perform some of the studies presented in Chapter 3. It was previously proposed that the holin of SPP1 would be encoded by *orf 26*. Its deduced product (gp26) shares homology and hydrophobic characteristics with the XhlB protein, which was implicated in the holin function of the cryptic phage PBSX of *B. subtilis*. However, our analysis revealed the upstream *orf 24.1* that encodes a holin-like protein analogous to XhlA, also involved in PBSX-mediated lysis. Therefore, we

decided to study the role of gp24.1 and gp26 as possible SPP1 holins. Because of its potential toxicity, holins of Gram-positive systems are often studied in heterologous systems, typically *E. coli*. However, despite some advantages, it is sometimes difficult to extrapolate the results obtained to the native systems. Thus, to understand the contribution of gp24.1 and gp26 to the SPP1holin function, the corresponding *orfs* were cloned separately and as a transcriptional fusion in a *B. subtilis* replicative plasmid, under the control of an inducible promoter. The results showed that in the assay conditions the individual production of these proteins did not produce significant impact on *B. subtilis* cell growth, despite their insertion and accumulation in the CM. Growth cessation and cell death typical of the holin action were only observed after co-production of gp24.1 and gp26, suggesting that in SPP1 the holin function may involve the production of these two proteins. Surprisingly, a constitutive promoter was identified within *orf 24.1*, which we believe should correspond to the previously described early promoter *PE5*. The presence of this promoter raises questions regarding lysis regulation in phage SPP1 (see Chapter 4).

Keywords: endolysin, holin, membrane potential, pmf, *B. subtilis*, SPP1, *S. aureus*, chimerical endolysin, antibiotic resistance.

ABBREVIATIONS

(those not defined in the text)

Ala	Alanine
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
CCCP	carbonylcyanide-*m*-chlorophenyl-hydrazone
CDD	Conserved Domain Database
DNA	Deoxyribonucleic Acid
DTT	Ditiotreitol
EDTA	Ethylenediaminetetraacetic acid
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D-thiogalactoside
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
OD	Optical Density
ON	Overnight
orf	Open Reading frame
PCR	Polymerase Chain Reaction
Pfam	Protein family
RNA	Ribonucleic Acid
SDS-PAGE	Sodium Dodecyl Sulphate - Poli-Acrilamide Gel Electrophoresis
SVM	Support Vector Machine algorithm

CHAPTER 1.
GENERAL INTRODUCTION

1.1 Bacteriophages: general features and life cycle

Bacteriophages, or phages, are viruses that specifically infect bacteria and were first described, independently, by Frederick Twort in 1915 (Twort, 1915) and Felix d'Herelle in 1917 (D'Herelle, 1917). In the Earth's biosphere they are the predominant biological entity estimated in more than 10^{31} particles. The phage population is also highly dynamic, with a rapid turnover occurring within a relatively short period of time, with 3.7 x 10^{30} phages being produced every year only in the deep hot biosphere (Breitbart *et al.*, 2004).

A phage classification system was first proposed by Bradley in 1967, recognizing six basic groups, in agreement with the type of nucleic acid, single (ss) or double (ds) stranded DNA or RNA, and gross morphology (Bradley, 1967). Nowadays, this scheme was adapted by the International Committee for Taxonomy of Viruses (ICTV) and archaeal viruses are classified into 15 families or corresponding groups, while those from Eubacteria belong to 10 families (Ackermann, 2012; King *et al*., 2012). Over 96% of the phages described in the literature are tailed and contain a genome of linear dsDNA enclosed in an icosahedral head or capsid, comprising the order *Caudovirales* (Ackermann, 2011). This order includes 3 families characterized according to the morphological features of the tail: 61% are *Siphoviridae* (long, non-contractile tails), 25% *Myoviridae* (contractile tails) and 14% *Podoviridae* (short tail) (Fig. 1.1) (Maniloff, 2012). The remaining families represent 4% of the studied phages, including polyhedral, filamentous and pleomorphic phages (Maniloff, 2012).

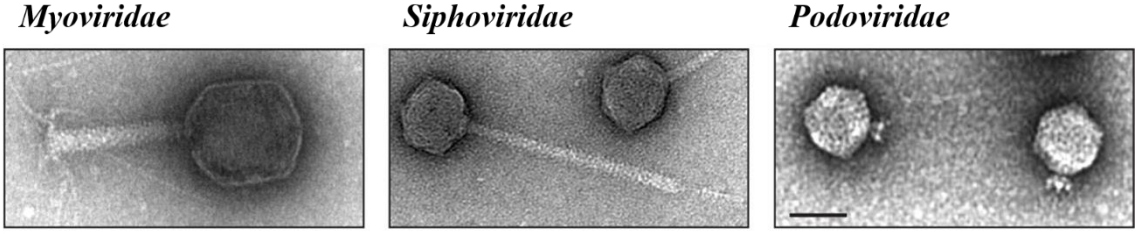

Fig. 1.1. Phages of the order Caudovirales. Transmission electron micrographs of a T4-like virus, HK97 and P22 are shown to represent the families *Myoviridae*, *Siphoviridae* and *Podoviridae*, respectively. Adapted from Krupovic *et al*. (2011).

According to their lifestyle, phages can be classified as virulent (or strictly lytic), when they replicate only by means of a lytic cycle, or as temperate when they have the possibility to follow either a lytic or a lysogenic pathway of development. Both cycles are initiated with virus particle attachment to the bacterial host cell, a process classically known as phage adsorption. This occurs through specific interactions between virion receptor binding proteins (RBPs) and receptors on the host cell surface (Vinga *et al*., 2006). Adsorption can take place in two steps. In a first step, the contact with bacterial surface may lead to reversible adsorption of the virus, with phages being released from the cells as infectious particles. This is a dynamic process that usually facilitates the second step, the irreversible adsorption, where phages become irredeemably committed to infection. As result of this irreversible interaction, the virion structure suffers a series of conformational changes that culminate in the injection of the viral nucleic acid into the host cell cytoplasm (Fokine and Rossmann, 2014; Maniloff, 2012; Molineux and Panja 2013; Vinga *et al*., 2006).

In the lytic cycle, phages like T4 immediately takeover the cellular machinery to express their genomes for phage nucleic acid replication and production of viral proteins. These are used to assemble the virion progeny that in the end is released through lysis of the infected cell (Kutter *et al* 2005; Maniloff, 2012). Under certain physiological conditions, temperate phages can follow the lysogenic pathway in a fraction of the infected cell population. In this case the virus maintains a stable relationship with the host cell and propagates its genetic material along with that of the bacterium, being passed to daughter cells during cell division. A common form of establishing lysogeny (e.g. phage λ) involves the integration of the viral genome into the host's chromosome, after which the integrated phage DNA is called prophage. Bacteria hosting a prophage are said lysogenic. Under some stress conditions, the prophage can be excised from the bacterial genome and "switch" to the lytic cycle (Kutter *et al* 2005; Maniloff, 2012).

1.2 Bacterial cell envelope: barrier to virus entry and release

The bacterial cell envelope is a complex multilayered structure that serves as the first and major line of defense against threats from the unpredictable and often hostile environment. It has the key function of maintaining cell homeostasis, providing at the same time structural and cell shape integrity by counteracting the high internal osmotic

pressure. It is also a remarkable physical barrier that phages need to overcome during the infectious cycle, firstly in order to transport their genome into the bacterial cytoplasm and in the end to release the viral offspring. The minimal components of the bacterial cell envelope are a cytoplasmic membrane (CM) and a cell wall (CW). Gram-negative bacteria and mycobacteria have in addition an outer membrane (OM) (Fig. 1.2).

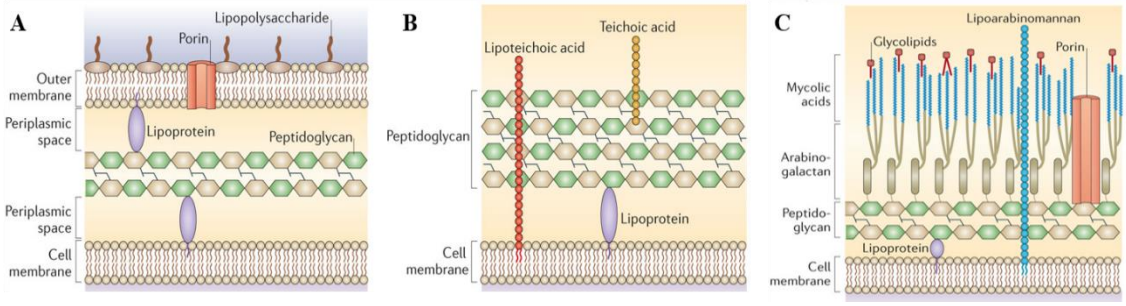

Fig. 1.2. Schematic representation of the bacterial cell envelope. (A) Gram-negative bacteria. (B) Gram-positive bacteria. (C) Mycobacteria. Adapted from Brown *et al*., 2015.

The CM is a phospholipid bilayer with embedded proteins (Singer, 1972) that isolates the cytoplasm and cellular components from the exterior. It is common to all bacteria and plays a central role in maintaining ion and solute gradients, which are essential to produce energy. It is the difference in the concentration and charges of ions on opposite sides of the CM that generates, respectively, the electrical potential ($\Delta\Psi$) and the chemical potential (pH gradient, ΔpH), the two components of proton motive force (pmf). The pmf is involved in the generation of ATP, control of bacterial autolysis, glucose transport and chemotaxis (Harold and Van Brunt, 1977; Nicholls and Ferguson, 1992). Any treatment that reduces either or both components of the pmf is said to depolarize the cell, which depending on the magnitude may affect cell viability (Novo *et al.*, 1999). The importance of pmf during phage infection will be discussed in more detail in section 1.3.

Bacteria do not lyse when put into distilled water because they have a rigid exoskeleton, the CW, which is at the same time sufficiently dynamic to allow maintenance of cell shape, cellular growth and cellular division (Archibald *et al*., 1993). The CW is mainly composed of peptidoglycan (PG), a polymer having as repeating unit a disaccharide made of N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM), linked by glycosidic bonds β ($1 \rightarrow 4$). The glycan chains are cross-linked by penta/tetrapeptide side stems that

are attached to NAM via amide bonds (Vollmer, 2008). Typically, the peptide stems are interconnected by an interpeptide bond in Gram-negative bacteria and by an interpeptide bridge of varying amino acid composition in Gram-positive bacteria (Labischinski and Maidhof, 1994). The amino acid sequence of stem peptides and of their cross-bridges accounts for more than 100 different PG types (Schleifer and Kandler, 1972; Vollmer, 2008).

In Gram-positive bacteria the CW is a multilayered network of cross-linked PG ranging between 20 and 40nm in thickness, whereas in Gram-negative cells the PG layer is much thinner (2-6nm) (Labischinski and Maidhof, 1994). This heteropolymer is the most conserved component of the cell envelope in bacteria, making it a major target for clinically used antibiotics, such as penicillin and vancomycin (Silhavay *et al*., 2010), and for phage-encoded PG hydrolases, such as endolysins, which act at the end of the lytic cycle to lyse infected cells.

The PG layers of Gram-positive bacteria are most often heavily modified with phosphate-rich molecules, the teichoic acids (TAs). These can be grouped into lipoteichoic acids (LTAs), which are anchored to the CM and extend through the PG layer, and wall teichoic acids (WTAs), which are covalently attached to the NAM residues of the PG and extend through and beyond the CW (Neuhaus and Baddiley, 2003; Weidenmaier and Peschel, 2008). Although their structures may be highly variable between species or even strains (Weidenmaier and Peschel, 2008), frequently the WTA backbones are polymers of glycerol phosphate (e.g., in *B. subtilis* 168) or ribitol phosphate (e.g., in *S. aureus* and *Listeria monocytogenes*) (Xia and Peschel, 2008), which are often modified and substituted with different sugars or amino acids, such as α-glucose and D-alanine (Neuhaus and Baddiley, 2003). Alone, WTAs can account for as much as 60% of the total CW mass, varying among organisms (Swoboda *et al*., 2010). LTAs, which are attached to the CM via a glycolipid anchor show less structural diversity than WTA, being usually formed by glycerol phosphate repeating units (Weidenmaier and Peschel, 2008). Together with PG, TAs make up a polyanionic matrix that plays a role in cation homeostasis, in trafficking of nutrients, proteins and antibiotics, and in the regulation of autolysins (see section 1.4) and presentation of envelope proteins (Neuhaus and Baddiley, 2003, and references therein). Even the Gram-positive bacteria lacking these polymers generally have functional anionic analogues, such as the lipomannan in *Micrococcus luteus* (Owen

and Salton, 1975; Powel *et al*., 1975). Bacterial strains with combined mutations that impair the expression of LTAs and WTAs are not viable (Oku *et al.*, 2009; Schirner *et al.*, 2009). *B. subtilis* and *S. aureus* mutants deficient in LTA biosynthesis can be obtained but are temperature sensitive and present several growth defects. WTA mutants also manifest increased sensitivity to temperature and certain buffer components, having the tendency to aggregate in culture (Oku *et al.*, 2009; Schirner *et al.*, 2009).

Also imbedded in the PG meshwork are sugar polymers, proteins and lipoproteins (DiRienzo *et al*., 1978). Many of these components are employed by phages to adsorb to their host cells. Mutations affecting the pathway of poly(glycerolphosphate) glucolysation have been associated with *B. subtilis* resistance to several phages such as ϕ25, ϕ29 and SP01(Yasbin *et al.*, 1973) and in reducing the infectivity of SPP1 (Baptista *et al.*, 2008).

The presence or absence of an OM is one of the major differences between Gram-negative and Gram-positive bacteria (Fig. 1.2). In Gram-negative the OM performs some of the functions attributed to Gram-positive TAs and in addition it establishes a compartment, the periplasm, which retains extracytoplasmic enzymes required for CW growth and degradation (DiRienzo *et al*., 1978). The OM is covalently linked to the thin layer of PG and serves as an additional stabilizing and protective barrier of the cell controlling the traffic of solutes. It is a lipid bilayer of asymmetrical composition, with the outer leaflet being composed of glycolipids, principally lipopolysaccharides (LPS), and the inner leaflet of phospholipids. The OM also contains integral proteins and lipoproteins (Kamio and Nikaido 1976; Ruiz *et al*., 2006).

The structure of the mycobacteria cell envelope is much more complex at the OM level. The mycobacterial OM presents a notable feature as in its inner side a mycolic acid-rich double layer is covalently attached to a layer of arabinogalactan, which in turn is covalently linked to the peptidoglycan that surrounds the CM (Hoffman *et al*., 2008). The outermost leaflet is composed of various glycolipids, including trehalose mono- and dimycolate phospholipids and species-specific lipids (Hoffmann *et al*., 2008; Zuber *et al*., 2008). Outside of the mycobacterial OM is a layer of proteins, polysaccharides and a small amount of lipids known as the capsule (Lemassu & Daffé, 1994; Lemassu *et al*. 1996; Sani *et al*., 2010).

1.3 Phage release from infected cells

As opposed to filamentous phages, such as M13, which are released from their hosts by a secretion-related mechanism (Russel *et al*., 1997), phages are bacteriolytic by nature (São-José *et al*., 2003, 2007). The ability to induce bacterial lysis is crucial for survival and ecological fitness of lytic phages. The destruction of the host immediately stops the production of new virions, raising an interesting biological question: when to destroy the infected cell to release newly assembled phages? Delaying lyses would compromise the opportunity to infect new hosts whereas a premature burst would release none or too little descendants (Wang *et al.,* 2000).

Tailed phages with dsDNA genomes (e.g. phage λ) accomplish bacterial cell lysis through the concerted action of at least two phage-encoded products: a small hydrophobic protein called holin that oligomerizes in the CM until it induces formation of pores (holes), and an endolysin, which is an enzyme that acts by digesting the PG in the bacterial CW (Young & Wang, 2006; São-José *et al*., 2007; Catalão *et al*., 2013). In the so-called canonical lysis model holins form micron-scale holes, which are essential to allow passage of the cytoplasm-accumulated endolysin to the CW (Dewey *et al*., 2010; Savva *et al*., 2014).

1.3.1 Lysis players: holin/endolysin molecular diversity

Endolysins

As mentioned above endolysins are phage-encoded PG hydrolases that act in the last stages of the infectious cycle to release the newly formed virion progeny. Phages infecting Gram-positive hosts and mycobacteria typically produce endolysins with a modular structure (López, *et al*., 1997; Payne and Hatfull,, 2012), where the enzyme's N-terminal region generally exhibits one or two catalytic domains (CDs) responsible for PG cleavage, and the C-terminal domain has one to several motifs (often repeats) involved in cell wall binding (CWBD) (Fischetti, 2005; Loessner, 2005; Nelson *et al*., 2012). With a reduced number of known exceptions (Briers *et al*., 2007; Walmagh *et al*., 2012), phages

of Gram-negative bacteria produce smaller endolysins comprising a single CD and no CWBD.

Endolysin CDs can be classified according to their cleavage specificities as *N*-acetyl-*β*-D-muramidases (lysozymes), *N*-acetylmuramoyl-L-alanine amidases, *N*-acetyl-*β*-*D*-glucosaminidases (glucosaminidases), endopeptidases, and lytic transglycosylases (Young *et al*., 2000) (Fig. 1.3). Lysozymes, glucosaminidases and lytic transglycosylases act on the glycan strands, breaking the *β*-1,4 glycosidic bonds between NAM and NAG. Amidases cleave the amide bond connecting NAM to the the first residue of the peptide stem, typically an L-Ala. Endopeptidases cleave within or between the peptide stems. Most of the reported endolysins are classified as muramidases and amidases.

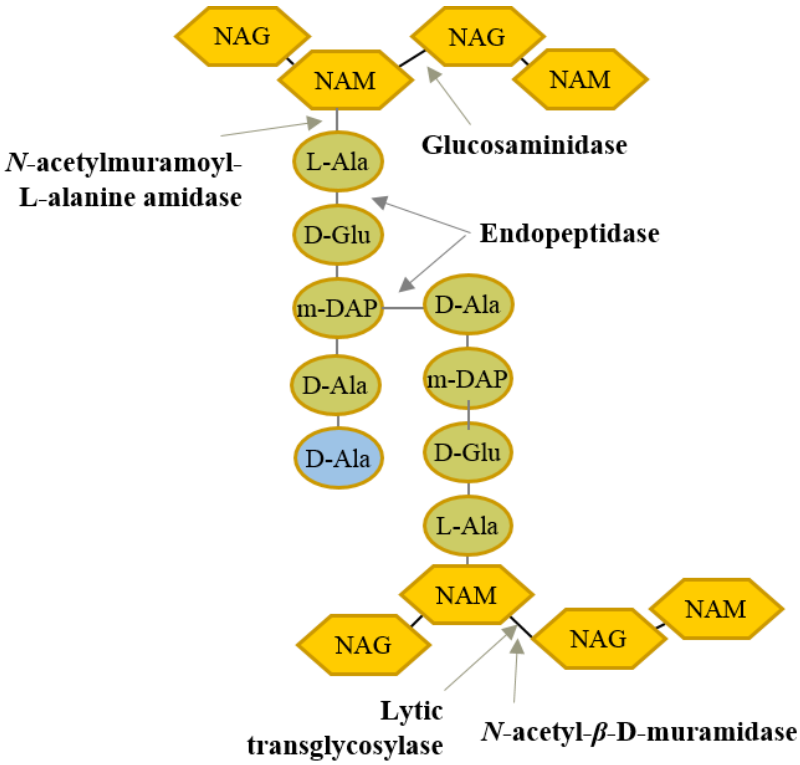

Fig. 1.3. Basic structure of peptidoglycan with indication of the bonds that are targeted by the five main types of enzymatic activities found in endolysins. m-DAP, which is found in the PG of *E. coli* and *B. subtilis*, is replaced by L-Lys in most Gram-positive bacteria. Linking between amino acid residues in positions 3 and 4 of adjacent stem peptides can occur via direct bonding or by an interpeptide bridge of variable amino acid composition. The D-Ala residue in blue is not always present.

It is important to note that most endolysin CDs and corresponding enzymatic specificities are identified solely based on homology and *in silico* analysis (Oliveira *et al*., 2013). Occasionally, the biochemical determination of endolysin cleavage products reveals enzymatic specificities different from those predicted by bioinformatics studies. Interesting examples are the streptococcal endolysins λSa1 and λSa2, which were initially predicted to have N- acetylmuramoyl-L-alanine amidase activity. However, electrospray ionization mass spectrometry analysis revealed that the endolysins displayed D-glutaminyl-L-lysine endopeptidase activity (Pritchard *et al*., 2007). In addition, the same CD family assigned *in silico* can specify different enzymatic activities. For instance the CDs of CHAP family can display amidase (Nelson *et al*., 2006) or endopeptidase (Navarre *et al*., 1999; Pritchard *et al*., 2004) activities, and there is at least one example where a single CHAP was shown to exhibit both activities (Linden *et al*., 2014). Nevertheless, nowadays there are several examples of endolysins that have been characterized by different biochemical methods, in addition to the examples provided above. These include the endolysins of *S. aureus* phages ϕ11 and K (Navarre *et al*., 1999; Becker *et al*., 2009), the endolysin of *Streptococcus agalactiae* phage B30 (Pritchard *et al*., 2004) and the *Listeria* phage endolysins Ply500 and Ply118 (Loessner *et al*., 1995).

A curious feature that is shared by many staphylococcal and streptococcal endolysins, including the enzymes referred to above, is the presence of two independent CDs with distinct catalytic activities. The endolysins of phages ϕ11 and K exhibit N-acetylmuramoyl-L-alanine amidase and D-alanyl-glycyl endopeptidase CDs (Navarre *et al*., 1999; Becker *et al*., 2009), the group B streptococcal B30 endolysin displays N-acetylmuramidase and D-alanyl-L-alanyl endopeptidase CDs (Pritchard *et al*., 2004), and the streptococcal λSa2 endolysin has a C-terminal N-acetylglucosaminidase CD in addition to the N-terminal endopeptidase domain (Pritchard *et al*., 2007). For other examples see the review Nelson *et al*., 2012.

Unlike CDs, CWBDs are much less conserved and this variability may be due to a selection made through evolution. Endolysins recognize not only single components of the CW but also those that are essential to the viability of the host cell, thus ensuring effective host cell lysis for liberation of the viral progeny. One of the first CWBD identified was that of the Cpl-7 endolysin encoded by the *Streptococcus pneumoniaee*

bacteriophage Cp-7, a lysozyme requiring choline or ethanolamine to achieve full activation (Garcia *et al*., 1990). Other common CWBDs in endolysins from Gram-positive systems are the motifs LysM and SH3 (with subtypes SH3b, SH3_3 or SH3_5) (Nelson *et al*., 2012; Oliveira *et al*., 2013). The former has been shown to bind the NAG residues of the PG (Ohnuma *et al*., 2008; Mesnage *et al*., 2014). SH3 CWBDs seem to target PG hydrolases to different components of the CW, including the PG peptide side chains and/or its cross bridges and also TAs (Loessner *et al*., 2002; Gründling and Schneewind, 2006; Lu *et al*., 2006; Tamai *et al*., 2014). Interestingly, although CWBDs are responsible for directing the endolysins to the CW, some studies seem to indicate that this is not always essential for lytic activity and for some endolysins their absence resulted even in increased lytic activity (Gaeng *et al*., 2000). However, other endolysins require both domains to maintain lytic activity at optimal levels (Loessner *et al*., 2002; Morita *et al*., 2001).

The generality of the endolysins of Gram-positive systems are, or are thought to be, monomeric products of a single gene. A remarkable exception is the pneumococcal endolysin PlyC that is composed by two different subunits, PlyCA and PlyCB, which are encoded by separate genes. PlyCA is a CD-containing polypeptide that associates with eight PlyCB subunits responsible for CW binding (Nelson *et al*., 2006; McGowan *et al*., 2012). Another exception is the two-component multimeric endolysin Lys170 that was shown to exhibit broad *in vitro* lytic activity against *Enterococcus faecalis* clinical strains (Proença *et al*., 2012). However, Lys170 subunits are produced from a single gene, due to the presence of an in frame internal translation start site that governs the independent production of the enzyme's CWBD (CWB170) (Proença *et al*., 2015a). Biochemical analysis indicated that Lys170 is composed of a full-length monomer of Lys170 associated with up to three CWB170 subunits (Proença *et al*., 2015a). Lys170 seems thus to exhibit an alternative strategy to increase the number of CWBDs, which was shown to favor the lytic activity of the endolysin. Alternative translation initiation (and eventually proteolytic processing) seems also to be on the genesis of multimeric endolysins in some clostridial phages (Dunne *et al*., 2016)

Endolysins must be distinguished from other phage-encoded PG hydrolases, the virion-associated lysins (VALs). These are integral components of the virion structure and are thought to locally digest the CW to facilitate injection of the phage genome into the host

cell (Rodríguez-Rubio *et al*, 2013). VALs generally lack a CWBD and in phages infecting Gram-positive bacteria they typically display two CDs, whereas those from phages infecting Gram-negative hosts usually have only one CD (Rodríguez-Rubio *et al*., 2013). In addition to the PG hydrolase activity, VALs frequently play a role in the assembly of the phage tail (Piuri and Hatfull, 2006; Boulanger *et al*., 2008). A reported exception to these features seems to be the VAL P17 of the staphylococcal phage 68, which shows the typical endolysin domain organization, that is, an N-terminal CD connected to a C-terminal CWBD (Takác *et al*., 2005). Curiously, a study performed by Rodríguez *et al*. (2011) showed that the two CDs of the VAL HydH5, encoded by the *S. aureus* phage phiIPLA88, had the ability to bind target cells. VALs may be part of different components of the tail, frequently tail knobs, tape measures, fibers or spikes (Moak and Molineux, 2000; Kanamaru *et al*., 2002; Kenny *et al.*, 2004; Piuri and Hatfull, 2006; Xiang *et al.*, 2008; Boulanger *et al*., 2008; Rodríguez-Rubio *et al*., 2012).

Holins

Holins constitute a very diverse functional group. Typically they are small hydrophobic proteins (<150 aa) with a hydrophilic and highly charged C-terminus (Wang *et al*., 2000; Young 2002). Holins and holin-like proteins are hole-forming transmembrane devices that mediate bacterial cell lysis during programmed cell death (PCD) or following phage infection (Reddy and Saier, 2013). A recent database survey and *in silico* analysis identified 52 holin families and representative members of these have been included in the Transporter Classification Database (TCDB; www.tcdb.org). Based on phylogenetic relationships, 21 one of the 52 families could be further grouped into 7 superfamilies, each with distinctive characteristics in terms of protein size, number of transmembrane domains (TMDs), probable membrane topology and organism source distribution (Reddy and Saier, 2013; Saier and Reddy, 2015).

In bacteria, holin-like proteins have been suggested to be responsible for the release of proteins from uninfected bacteria (Desvaux and Hebraud, 2006; Desvaux *et al*., 2005, 2009). The Gram-positive bacteria *Clostridium difficile*, the major cause of antibiotic-associated diarrhea, encodes two toxins, TcdA and TcdB within a pathogenicity locus (PaLoc) as well as the holin-like protein TcdE (Burdon *et al*., 1981). Govind and Dupuy (2012) showed that TcdE facilitates the release of both *C. difficile* toxins to the

extracellular environment. However, unlike most phage holins it did not cause the non-specific release of cytoplasmic contents. In other systems holin-like proteins are involved in the regulation of murein hydrolase activity and apoptosis of bacteria, as exemplified by the *S. aureus* CM-associated proteins CidA and LrgA, which resemble structurally and functionally holin and antiholin (see below) proteins, respectively (Ranjit *et al*., 2011). The regulation of operons *cid* and *lrg* will be detailed in section 1.4.2).

Regarding holins of phage origin, they were initially grouped into three main classes based on the number of TMDs: class I, with 3 TMDs, class II with 2 TMDs and class III with 1 TMD, as represented by the holins of *E. coli* phages λ, 21 and T4, respectively (Wang *et al*., 2000) (Fig. 1.4). However, the recent study referred to above that defined the 52 holin families (and 7 superfamilies) raised the possibility of holins with 4 TMDs and membrane topologies alternative to those depicted in Fig. 1.4. In addition, it was proposed for at least two phages that the holin functional unit may be a complex of two distinct holin-like proteins (see below).

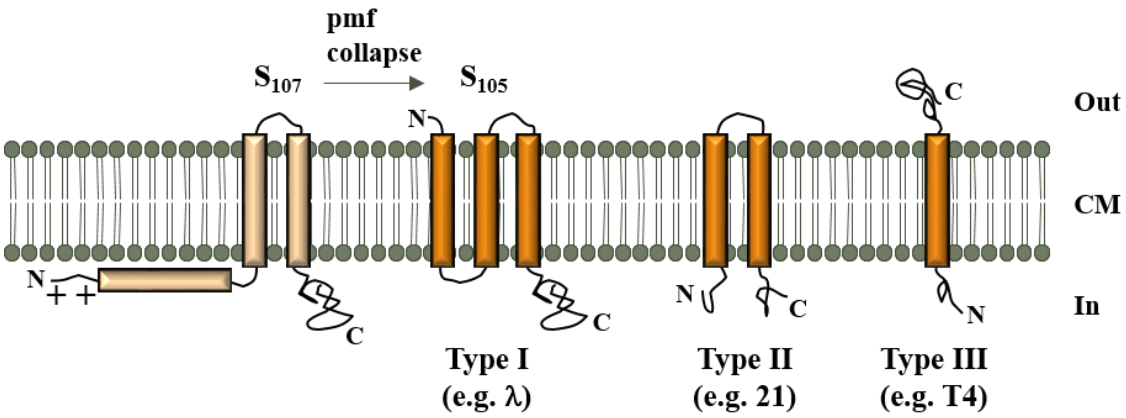

Fig. 1.4. Topological models of three classes of holins and of one antiholin. Holin classes (dark orange) are represented by the S_{105} holin of phage λ (class I, 3 TMDs), the S^{21} holin of phage 21 (class II, 2 TMDs) and the T holin of phage T4 (class III, 1 TMD). The topology of phage λ antiholin S_{107} (light orange) in an energized membrane is shown, where the first TMD does not insert in the CM due to two extra positively charged amino acids at the N-terminus. After pmf dissipation the first S_{107} TMD enters the CM and adopts a S_{105}-like topology, with the two proteins contributing to hole formation (see text for details). Adapted from São-José *et al*., 2007.

Holins have been described has having two main functions in the phage infection cycle: i) to determine the end of infection by means of its pmf-dissipation activity (the killing effect), and ii) allow the release to the CW of the cytosol-accumulated endolysins, since the majority of these lack secretory or export signals (the transport effect) (Catalão *et al*., 2013; Young, 2013, 2014). Such signals are nevertheless present in some endolysins, which are thus transported to the CW via a holin-independent pathway (see below). In these cases the holin killing effect seems crucial to fully activate the exported endolysins. In these systems holins may perform small-sized holes ("pinholes") and for that reason they are called pinholins (Pang *et al*., 2009, 2013; Park *et al*., 2007).

As long as the pmf is maintained above a certain threshold, holins progressively and harmlessly accumulate as mobile oligomers in the CM during the course of phage infection, until they reach an allele-specific critical concentration (To and Young, 2014; White *et al*., 2011; Young and Wang, 2006). After this point, holin nucleation and aggregation occurs with formation of few large rafts that are proposed to permeabilize the CM to protons and ions. The pmf dissipation is then thought to suddenly cause conformational changes that allow the holin rafts to convert into the final holes (To and Young, 2014; White *et al*., 2011). This event ends macromolecular synthesis and thus effectively terminates infection, reason way these proteins are considered the clocks of phage infection (Wang *et al*., 2000; Young *et al*., 2000).

To fine tune the host cell lysis timing some phages, notably the lambdoid phages, encode also a holin antagonist, referred to as antiholin. In phage λ the holin *S* gene itself, through the dual-start motif encodes both the lysis and inhibitor functions (Bläsi and Young, 1996). The S holin, or S_{105}, is a 105 aa polypeptide with 3 TMDs that adopts an N-out, C-in topology (Fig. 1.4; Gründling *et al*., 2000a). S_{105} results from translation initiation at the second start codon (Met_3) of the *S* gene. The antiholin S_{107}, which initiates at the first start codon (Met_1) of *S* differs from S_{105} by two residues at the N-terminus, Met and Lys, which confer to the antiholin N-terminus 2 extra positive charges (one charge from the deformylated Met_1 and another from the Lys_2 residue, White *et al*., 2010). These positive charges prevent insertion of the first TMD of S_{107} into the CM (Fig. 1.4; Gründling *et al*., 2000a). This, associated to the capacity of the antiholin to dimerize with S_{105} (Gründling *et al*., 2000b) is what confers to S_{107} its inhibitory effect. Quite remarkably, dissipation of the pmf triggers the insertion of the first antiholin TMD into

the CM, adopting thus the same topology of S_{105} and contributing similarly to hole formation (Young, 2002 and references therein).

The holin dual-start motif seems also to be present in some phages infecting Gram-positive hosts, like for example in phages ϕ29 and A118 of *B. subtilis* and *Listeria monocytogenes*, respectively (Tedin *et al*., 1995; Vukov *et al*., 2003). However, in the latter phage the dual-start motif in the holin gene *hol118* did not seem to be involved in the regulation of holin activity. Curiously, a third in frame and internal translation start was found further downstream in *hol118* (Met_{14}), which was shown to direct the synthesis of an inhibitor function of Hol118 (Vukov *et al*., 2003).

T4 also presents a regulatory paradigm in terms of holin function. The T4 holin T has all the characteristics presented by other canonical holins (Wang *et al*., 2003), however this protein can also respond to environmental stimulus, such as superinfection by other T4 virions. In this condition the infected cells engage in a LIN ("lysis inhibition") state where holin-mediated lysis is delayed (Doermann, 1948). T4 LIN state involves a complex of three proteins that span the cellular envelope, and which is composed by the holin T, the antiholin RI and the associated factor RIII (Chen and Young, 2016). After activation by superinfection, the protein RI is secreted to periplasm and binds to periplasmic domain of holin T blocking its lethal permeabilization of the CM (Dressman and Drake, 1999; Ramanculov and Young, 2001). Conversely, the RIII interacts with cytoplasmic domain of T, which blocks the membrane hole-formation (Chen and Young, 2016). This state can be subverted in the presence of energy poisons or if the superinfection decreases (Abedon, 1994; Tran *et al*., 2005).

It is relatively common to find in the genome of phages infecting Gram-positive bacteria two holin-like genes clustering with that of the endolysin (Sheehan *et al*., 1999; Catalão *et al*., 2013). Certainly inspired by the lambdoid lysis mechanisms, some authors have speculated that these genes might encode the antiholin/holin functions. At least for two phages however, this pair of holin-like proteins seems to be needed for efficient lysis of the infected cell. This has been shown for the holin-like pairs Gp4 and Gp5 of mycobacteriophage Ms6 (Catalão *et al*., 2011) and XhlA and XhlB of *B. subtilis* phage PBSX (Krogh *et al*., 1998). It was proposed in these cases that the holin "functional unit" could be a complex made of each pair of holin-like proteins.

Besides the holin/endolysin pair, in Gram-negative bacteria and mycobacteria other proteins proved to be important for efficient host cell lysis in certain conditions. The most studied examples are the Rz and Rz1 proteins of phage λ, a type II integral membrane protein and an OM lipoprotein, respectively (Hanych *et al*., 1993; Kedzierska *et al*., 1996). Both proteins interact through the C-terminal end of their periplasmic domains and form a complex that spans the periplasm, connecting the CM to the OM (Berry *et al*., 2010, 2013). This complex termed spanin accumulates in the cell envelope throughout the infectious cycle (Berry *et al*., 2010, 2013). In the lack of Rz/Rz1, after the infectious cycle, the host cell terminates in a spherical form (Young *et al*., 1979; Zhang and Young, 1999), in which the CM has been lethally permeabilized by the holin and the PG destroyed by the endolysin, but the OM is intact. The spanin complex is responsible for the elimination of this last barrier, most probably by promoting the fusion between the IM and OM (Rajaure *et al*., 2015). This final step can be essential for the efficient release of the virion progeny, which likely explains the broad dissemination of spanins among phages of Gram-negative bacteria (Summer *et al*., 2007).

Other phage proteins can be viewed as functional analogues of spanins, such as LysB from mycobacteriophages (Gil *et al*., 2008; Catalão *et al*., 2013) and LysB-homologues identified in phages infecting *Rhodococcus equi* (Summer *et al*., 2011). LysB from phage Ms6 of *Mycobacterium smegmatis* was shown to hydrolyze ester bonds in esterase and lipase substrates (Gil *et al*., 2008), cleaving the bonds between OM mycolic acids and the arabinogalactan of the mycobacterial cell envelope (Payne *et al*., 2009; Gil *et al*., 2010). It was proposed that LysB acts together with the endolysin LysA, degrading the mycolyl-arabinogalactan-peptidoglycan complex, thus ensuring efficient host cell lysis at the end of a phage infection cycle (Gil *et al*., 2010). As *R. equi* also has an OM composed of mycolic acids covalently linked to PG (Sutcliffe, 1998), it is legitimate to assume that the function of the LysB-homologues is the same as that described for LysB of MS6.

1.3.2 Lysis mechanisms in tailed phages

Endolysin release to the CW can absolutely depend on the holin-formed CM lesions or, less frequently, the enzyme can be actively transported by machinery of the host bacterium. In fact, apart from details specific of each system, known host cell lysis

mechanisms of tailed phages can grossly be divided in two major types, depending on whether endolysin transport is holin-dependent or -independent (Fig. 1.5).

In this thesis the designations canonical endolysin (c-endolysin) or exported endolysin (e-endolysin) will be used when referring to the enzymes that are holin-dependent or -independent for export, respectively. An interesting observation is that even phages encoding e-endolysins, to which a holin function could be thought as dispensable, also produce holin-like proteins (Schmidt *et al*., 1996; São-José *et al*., 2004; Frias *et al* 2009; Catalão *et al* 2011). In these systems, holins still maintain the key role of defining the proper time for lysis thanks to their scheduled pmf-dissipating action, which directly or indirectly relieves the mechanisms restraining lytic activity of e-endolysins (Xu *et al*., 2004, 2005; Nascimento *et al*., 2008; Sun *et al*., 2009; Catalão *et al*., 2010; Frias *et al*., 2013) (Fig. 1.5).

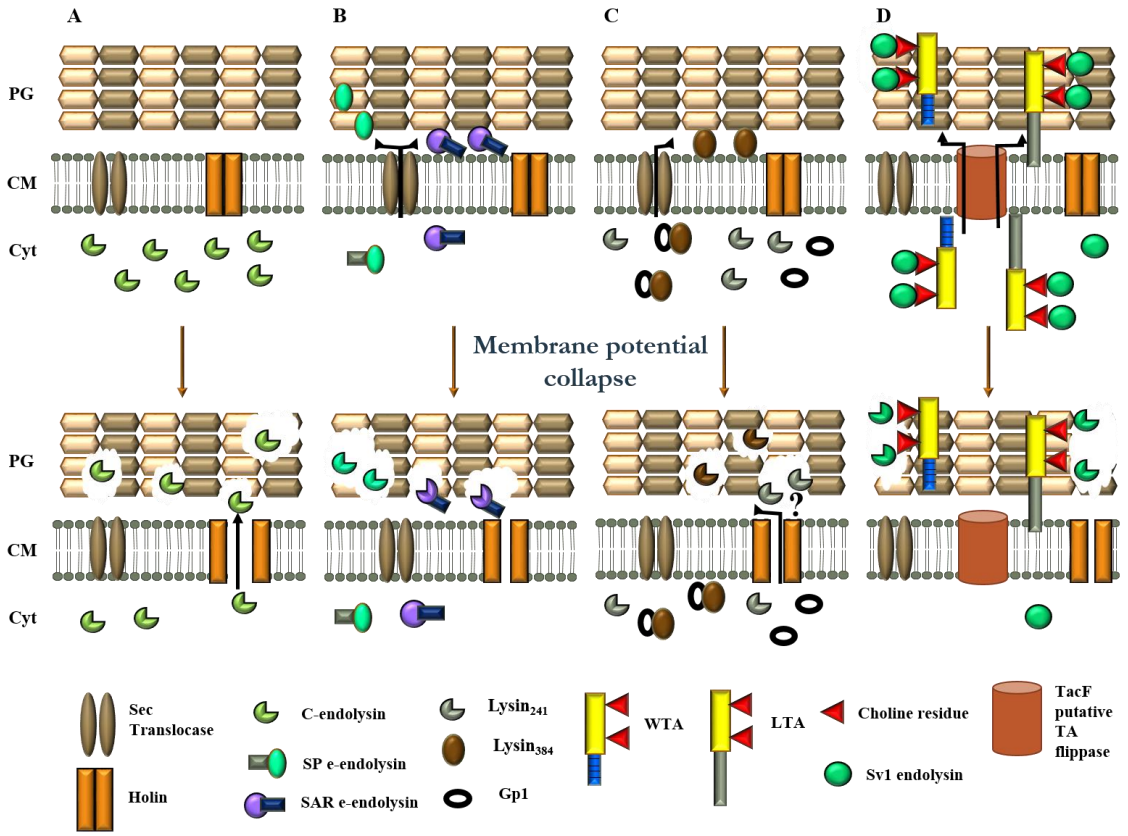

Figure 1.5. Models for export and activation of phage endolysins. C-endolysins reach the CW through the holin pores, as represented by phage λ **(A)**. E-endolysins are exported to the CW in a holin-independent manner. Export occurs through the Sec-system of the host bacterium when endolysins are produced with a typical signal peptide (SP) or with a signal-anchor-release (SAR) sequence, as observed in oenophage fOg44 and in coliphage P1, respectively **(B)**. In mycobacteriophage Ms6 the Sec-mediated export of the full-length endolysin $Lysin_{384}$ is assisted by the chaperone Gp1, whereas the truncated endolysin $Lysin_{241}$ is predicted to access the CW through the holin channels **(C)**. E-endolysins might be exported to the CW along with TAs precursors, in a holin- and Sec-independent way, as proposed for the pneumococcal phage SV1 **(D).** PG, peptidoglycan; CM, cytoplasmic membrane; Cyt, cytoplasm. Endolysins with the "Pacman" shape represents c-endolysins and e-endolysins activated after pmf collapse. Adapted from Catalão *et al*., 2013.

In the following sections the different lysis mechanisms employed by dsDNA tailed phages will be presented in more detail.

1.3.2.1 Holin-dependent export of endolysins

- **The model mechanism of *E. coli* phage λ**

The λ lysis cassette consists of four genes, *S*, *R*, *Rz* and *Rz1*. The *S* gene encodes the holin (S_{105}) and the antiholin (S_{107}) functions by the dual-start motif, the *R* gene the endolysin, and the Rz and Rz1 products compose the spanin function (see above) (Berry *et al*., 2008; Rajaure *et al*., 2015). In the phage λ lytic system, the prototype of the canonical lysis model, the holin accumulates in the CM during late gene expression without disturbing its integrity, while the enzymatically active endolysin builds in the cytoplasm. At a genetically-defined time, the membrane potential is dissipated and the holin triggers to form holes (Young, 2002 and references therein). Although these are lethal to the cell, complete lysis requires the action of the R endolysin (Young *et al*., 2000). At the time of its lethal triggering the S holin exists in large aggregates, forming lesions in the CM that are large enough to allow access of the endolysin to the CW. Once in this cell compartment, the lytic enzyme rapidly cleaves the PG substrate, which is thought to facilitate IM and OM fusion by the Rz/Rz1 spanin complex, finally leading to host cell lysis (Dewey *et al*., 2010; White *et al*., 2011; Rajaure *et al*., 2015).

1.3.2.2 Holin-independent export of endolysins

- **Phage endolysins that engage the bacterial Sec-system for their targeting to the CW**

***Oenococcus oeni* phage fOg44 and its endolysin with a signal peptide.** A lysis strategy deviating from the λ paradigm was first described in the temperate phage fOg44, which infects the Gram-positive bacterium *O. oeni*. The fOg44 endolysin Lys44 was shown to be equipped with a typical signal peptide (SP) sequence, which engages the general secretion pathway of bacteria (the Sec system) for its transport to the CW, with the subsequent removal of the SP after export to produce the mature and active endolysin (São-José *et al*., 2000). The active Lys44 is detected half-way through the phage latent period, accumulating progressively until cell lysis. This indicates that the endolysin is targeted to the CW from the moment of its synthesis (São-José *et al*., 2000). Although at first sight dispensable, the fact is that fOg44 also produces a functional holin (Hol44)

(São-José *et al*., 2004). The transcription of *lys44* and *hol44* during fO44 lytic growth appears to result in a stable dicistronic mRNA (Parreira *et al*., 1999), suggesting a concomitant production of both lysis products during phage infection as observed in λ (São-José *et al*., 2007). To understand the holin role in this lytic system, Nascimento *et al*. (2008) performed an elegant study in which efficient sensitization of metabolically active cells to Lys44 action was only achieved with agents that promoted the non-selective permeabilization of the CM to ions (in this case nisin and chloroform), i.e., agents that mimic the holin disruption of the CM electrical and chemical gradients. These results revealed that even in these strategies the lysis timing is determined by the holin-mediated dissipation of the pmf, which should activate the e-endolysins pre-localized in the CW.

Phage endolysins with SAR sequences. The endolysins Lyz^{P1} and R^{21}, produced by *E. coli* phages P1 and 21, respectively, have in their N-terminus an unusual TMD characterized by the presence of weakly hydrophobic amino acids, flanked by charged residues (Xu *et al*., 2004). This domain currently known as signal-anchor-release (SAR) sequence is not cleaved as the SP of Lys44, although it also enables the endolysin export through the host Sec system (Xu *et al*., 2004, 2005). As for Lys44, a model based on pmf disruption was proposed to explain the triggering of lysis with SAR endolysins. In this model the holin accumulates in CM without affecting the pmf, whereas the endolysin is secreted but retained in an inactive form, tethered to the CM. At the programmed lysis time the holin triggers, disrupting the membrane and leading to pmf abolishment. This depolarization promotes release of the SAR sequence from the membrane, which results in endolysin refolding into its catalytically active form (Xu *et al*., 2004, 2005; Sun *et al*., 2009). This functional regulation is essential to avoid premature lysis of the infected host. For phages encoding SAR endolysins, the holin protein needs only to produce lesions large enough to allow passage of ions and depolarize the CM, in order to fulfill its role in controlling the timing of lysis. In fact, it was demonstrated that the holes formed by some cognate holins (e.g. S^{21}) of SAR endolysins are too small to allow passage of the λ endolysin (Park *et al*., 2007). Detailed structural and biochemical studies showed that instead of the micron-scale holes formed by the S holin of λ, S^{21} formed channels with a lumen estimated at ~2 nm (Pang *et al*., 2009). For this reason the term "pinholin" has been proposed to differentiate the small-hole (pinhole)-forming character of the phage 21 holin from the canonical holins that form large holes (Park *et al*., 2007).

Endolysin export mediated by a chaperon in mycobacteriophage Ms6. In addition to the predicted endolysin (LysA) and holin (Gp4), the temperate *M. smegmatis* phage Ms6 encodes three additional proteins within its lysis module: Gp1, LysB and Gp5 (Garcia *et al*., 2002). An interesting novel feature in this mycobacteriophage is that Gp1 is a chaperon-like protein that interacts with LysA and promotes its export to the CW in a holin-independent way (Catalão *et al*., 2010). A deletion of *gp1* from the Ms6 genome revealed that, although not essential for plaque formation, the chaperone is necessary for the phage to achieve an efficient lysis, with its absence resulting in a decrease of ~70% in the burst size. Curiously, experiments in *E. coli* showed that lysis could be prevented in mutants of the Sec system, even with the concomitant expression of Gp1 and LysA, indicating an involvement of this bacterial secretion system also in LysA export (Catalão *et al*., 2010). As referred previously, the protein LysB is probably an auxiliary agent in lysis that functions analogously to spanins of Gram-negative phages.

Ms6 lysis regulation seems also to display other peculiar features. Gp4 was described as a class II holin, with the first TMD having SAR features, followed by a typical TMD (Catalão *et al*., 2011). Results suggest that Gp4 forms pores too small to allow passage of the 43-KDa Ms6 endolysin, thus behaving as a pinholin. A second putative holin gene (*gp5*), encoding a protein with a single predicted TMD at the N-terminal region, was also identified at the end of the Ms6 lytic operon. Neither the putative class II holin nor the single TMD polypeptide could trigger *E. coli* lysis when produced in pairwise combinations with the endolysin LysA (Catalão *et al* 2011). However, cross-linking experiments showed that Gp4 and Gp5 oligomerize and that both proteins interact, suggesting that the correct and programmed timing of lysis is achieved by the combined action of Gp4 and Gp5 (Catalão *et al* 2011).

As referred for the other secreted endolysins, holin function in Ms6 seems also to be confined to the regulation of lysis timing through endolysin activation. Indeed, co-expression of Gp1 and LysA in *M. smegmatis* caused only partial lysis, but the addition of nisin led to complete lysis (Catalão *et al*., 2010). The authors suggested that the endolysin was already positioned next to its target, the PG, at lysis onset, since the pore diameter produced by nisin (2.5 nm) should not allow the passage of a protein as large as the Ms6 endolysin (Ruhr and Sahl, 1985). Accordingly, the authors also envisaged that the Ms6 holin needs only to depolarize the mycobacterial CM to accomplish its role in

controlling the timing of lysis (Catalão *et al.*, 2010). However, how the Ms6 endolysin is kept inactive until the holin trigger is a question that remains to be elucidated (Catalão *et al.*, 2013).

- **SV1 phage, a new type of endolysin transport?**

Recently, a new mechanism of endolysin transport to the CW was proposed for phage SV1 that infects the Gram-positive bacterium *S. pneumoniaee*. In this system, endolysin transport does not rely on the holin function and it is yet to prove the involvement of the Sec system. Phage SV1, like all known phages infecting *S. pneumoniae* has a typical endolysin (Svl) that lacks any recognizable secretory signal such as SAR or SP (López and García, 2004). Therefore, until recently, it had been proposed that endolysin release to the PG occurred as described in the canonical holin-endolysin model (Haro *et al.*, 2003; López and García, 2004; Martín *et al.*, 1998). However, a recent work performed by Frias *et al.* (2013) demonstrated that Svl export and anchoring to the cell surface is most likely dependent on the presence of choline residues in the TAs of the CW. Choline is metabolized to decorate the TAs chains (Tomasz, 1967), serving as an anchor for a family of choline binding proteins (CBPs). These surface exposed CBPs are involved in the virulence and pathogenesis of *S. pneumoniaee* (Gosink *et al.*, 2000; Rosenow *et al.*, 1997). In the absence of choline, cells grow in chains and fail to lyse in the stationary phase due to the lack of activity of the septum-cleaving LytB glucosaminidase and of the autolysin LytA, both of which are CBPs (Tomasz *et al.*, 1971). Interestingly, the authors noted that as already observed for LytA (Díaz *et al.*, 1989), the phage endolysin Sv1 was not found in the CW when host cells were grown in the presence of ethanolamine, which is used to replace choline in TAs (Ware *et al.*, 2005). Thus, although the exact mechanism by which Sv1 is translocated to the CW is still unknown, the high homology observed between the endolysin and LytA (Romero *et al.*, 1990) lead the authors to speculate that both proteins may share the same export pathway (Frias *et al.*, 2013). Since choline anchors the lytic enzymes to the CW, it was hypothesized that they could bind intracellularly the TA precursors loaded with choline and then be co-transported across the CM by the TA biosynthetic machinery (Frias *et al.*, 2013). As observed for other e-endolysins, it is the pmf disruption achieved by the holin function, or by pmf-dissipating agents such as DCCD, the key factor for Sv1 endolysin activation (Frias *et al.*, 2013).

1.4 Lysis regulation: insights from autolysis in Gram-positive bacteria

Regardless the lysis strategy used by tailed phages, it is clear that the moment of killing and burst of the infected cell must be tightly regulated to avoid premature end of the virus reproductive cycle. The regulation of lysis functions in time and/or space can be imposed at the transcription, post-transcription, translation and post-translation levels (for a detailed review on the possible stages at which regulation of lysis functions can occur see Pohane and Jain, 2015). Relevant to the work presented in this thesis are the post-translation mechanisms acting on endolysins, firstly on the regulation of their translocation across the CM (which can be either holin-dependent or –independent), and then after reaching the CW compartment.

One aspect that is common to the already discussed lysis strategies is the killing of the infected cell through the holin pmf-dissipation action, regardless of how endolysins are exported to their site of action. It is well known for many bacteria that lysis can be a secondary response to cell death, in the so-called autolytic phenomenon, i.e., autolysis is a *post-mortem* effect (Smith *et al*. 2000). Considering the high level of homology that can be observed between phage endolysins and bacterial autolysins (López and García, 2004; Oliveira *et al*., 2013), it seems legitimate to considerer that the same factors that regulate autolysins might also be involved in the regulation of the activity of endolysins during phage development, especially in the case of e-endolysins (São-José *et al*., 2000, 2003).

Autolysins are bacterial PG hydrolases that form a vast and highly diverse group of enzymes capable of degrading PG, in a similar way as described for endolysins (Fig. 1.3). These enzymes are universal among bacteria that possess PG and due to their potential lethality they are under a tight control. Beyond autolysis, autolysins are also involved in numerous cellular processes including cell division, CW turnover, PG maturation, cell elongation, motility, chemotaxis, genetic competence, protein secretion, differentiation and pathogenesis (Foster, 1994; Blackman *et al*., 1998).

B. subtilis, a bacterial species central to the work presented in this thesis (see next chapters), encodes at least 35 differentially regulated autolysins, clustered into 11 families on the basis of amino acid sequence similarities (Smith *et al*., 2000). The major autolysins present during vegetative growth are LytC, LytD and LytF (Blackman *et al*., 1998; Smith

et al., 2000). LytC is an amidase that localizes uniformly to the CW, (Yamamoto *et al*. 2003), being required for efficient swarming motility, cell separation and CW turnover, and it is the major PG hydrolase involved in autolysis of *B. subtilis* (Kuroda and Sekiguchi, 1991; Blackman *et al*., 1998; Smith *et al*., 2000). LytD is an N-acetylglucosamine, whose CD is homologous to the corresponding CD of the bifunctional autolysin Atl of *S. aureus* (Foster, 1995). LytD seems to cooperate with LytC in the same cellular processes (Smith *et al*., 2000). Curiously, under certain conditions the absence of LytD does not seem to lead to obvious cell phenotypes (Margot *et al*., 1994). LytF is a member of DL-endopeptidase II and, as LytE, it localizes to the division septa and contributes to vegetative daughter cell separation (Margot *et al*., 1998; Smith *et al*., 2000).

1.4.1 The phenomenon of bacterial autolysis: first dissipate pmf and then lyse

In *B. subtilis* there are many studies describing that autolysis is triggered after treating cells with different membrane-depolarizing agents, such as gramicidin, monensin (selectively disrupts ΔpH), valinomycin (disrupts only $\Delta\psi$), CCCP and energy poisons like sodium azide (Chung *et al*., 2009; Joliffe *et al*., 1981). It has also been observed that lack of oxygen in *B. subtilis* cultures enhances autolysis and when these become static, cell lysis progressively occurs within few hours (Joliffe *et al*., 1981 and references therein). Autolysis is also triggered after brief starvation for a carbon source. Interestingly, the addition of electron-donating agents (e.g. phenazine methosulfate or ascorbate) or carbon sources (e.g. glucose or glycerol) to lysing bacteria retards or inhibits autolysis (Joliffe *et al*., 1981). Additionally, it was demonstrated that when applied exogenously the peptide toxin SDP rapidly collapses the pmf, presenting as secondary effect the trigger of the slow process of autolysis (Lamsa *et al*., 2012). Indeed, SDP was proved to cause cell death without lysis in *B. subtilis* strains defective in autolysins LytC, LytD, LytE and LytF (Lamsa *et al*., 2012). In conclusion, membrane energy and autolysis are tightly coupled in *B. subtilis*.

In *S. pneumoniaee*, the collapse of the membrane pmf induced by the holin of phage SV1 was shown to activate not only the phage endolysin Svl, but also the autolysin LytA (Frias *et al*., 2009, 2013). The holin of the pneumococcal phage EJ-1 seemed also capable to activate LytA and cause cell death when the proteins where co-produced in *E. coli* (Díaz *et al*., 1996). A similar phenomenon is probably induced by *B. subtilis* phage PBSX, since

the presumable XhlA/XhlB complex responsible for the holin function was shown to induce bacterial lysis, through autolysin activation, in the absence of the phage endolysin XlyA (Krogh *et al*., 1998). These results support the previous speculation that, at least in Gram-positive systems, the holin-mediated pmf dissipation could also activate the bacterial autolytic machinery, which is known to be turned on upon CM injury (São-José *et al.*, 2000, 2003, 2007).

Bacteriocins that cause lesions in the CM of sensitive cells due to their small size, high hydrophobicity and predicted amphipatic α-helixes (Hauge *et al*., 1998) are also capable of inducing autolysis in some *Lactococcus* and *Lactobacillus* strains (Martínez-Cuesta *et al*., 2000). Again, bacterial lysis is a consequence of bacteriocin-induced depletion of the cellular energy (Martínez-Cuesta *et al*., 2000) and it is worth noting that the biophysical properties of some of these agents resemble those of phage holins (São-José *et al*., 2003).

The β-lactam and glycopeptides antibiotics (e.g. penicillin and vancomycin, respectively) inhibit proper CW synthesis at different stages of the process, leading to bacterial cell lysis (Ruzin *et al*., 2004). However, it was shown for some bacteria that such lysis is preceded by cell death, which in fact activates the autolytic enzymes. This was observed with the *S. pneumoniaee* autolysin LytA after addition of penicillin, vancomycin and bacitracin (Tomasz *et al*., 1970; Tomasz and Waks 1975; Mellroth *et al*., 2012). The same phenomenon could be observed in *B. subtilis* with cloxacillin, a β-lactam antibiotic (Blackman *et al*., 1998). Interestingly, whereas the *B. subtilis* autolysins LytC and LytE mediate lysis in response to cell death induced by energy poisons, LytD appears not to respond to these stimuli (Margot *et al*., 1998).

The *S. aureus* operons *cid* and *lrg* are known to be involved in the control of bacterial PCD (Bayles, 2007). These operons encode the predicted membrane-associated proteins CidA, CidB, LrgA and LrgB, all containing multiple TMDs (Groicher *et al*., 2000; Rice *et al*., 2003). Their expression is regulated by LytSR, a two-component regulatory system also involved in murein hydrolase activity and tolerance to penicillin (Brunskill and Bayles, 1996**;** Groicher *et al.*, 2000). A study performed by Ranjit *et al*. (2011) lend support to the early proposals (Groicher *et al*., 2000; Rice *et al*., 2003) that CidA and LrgA are analogous to the λ holin and antiholin proteins, respectively, functioning in the control of cell death and lysis during biofilm formation. A mutation in the *lrgAB* operon

resulted in increased PG degradation and its overexpression caused increased penicillin tolerance in both *lrgAB* mutants and parental strains during early exponential phase (Groicher *et al*., 2000). Conversely, a *cidA* mutant had decreased PG hydrolase activity when compared with the parental strain and a high tolerance to antibiotics such as penicillin, rifampicin and vancomycin (Rice *et al*., 2003; Rice *et al*., 2005). This regulatory system is activated by the collapse of pmf, specially the $\Delta\psi$ component. When this occurs the transcription of the *lrg* operon increases and lysis occurs, probably because LrgA converts into a holin function, after pmf dissipation, as it happens with λ S_{107} (Patton *et al*., 2006). Additionally, it was demonstrated that transcription of the *cid* operon was unaffected by pmf alterations, being instead activated by the presence of acetic acid (Patton *et al*., 2006). The *cid* operon plays also an important role in biofilm development as its mutation leads to biofilms loosely compacted and less adherent to the substrate (Rice *et al*., 2007).

The above described *cid* and *lrg* regulatory system is widely conserved in bacteria but it also shares interesting similarities with the Bcl-2 family of proteins, which are involved in the regulation of apoptosis in eukaryotic cells (Bayles, 2003). Mitochondrial permeabilization is controlled by Bcl-2 proteinsthat include the antiapoptotic protein Bcl-2 and the proapoptotic protein Bax. In response to apoptotic signals the mitochondria undergo OM permeabilization releasing cytochrome c and other cytoplasmic proteins (Kromer *et al*., 2007). An experiment performed by Pang *et al*. (2011) showed that when Bax is expressed in bacteria it displays holin like-behavior, causing endolysin release and cell lysis in a process dependent on Bax oligomerization. Like antiholins, the interaction of Bcl-2 with Bax inhibited the induction of cell death (Kroemer *et al*., 2007).

1.4.2 Autolysis regulation

The autolysis phenomenon in bacteria is influenced by a variety of factors such as ion and carbon source content of the medium (e.g. NaCl and glucose), pH, growth phase, proteases, cardiolipin, TAs, temperature and oxygen (Rice and Bayles, 2008 and references therein). Although the role of pmf in autolysis control seems safely established, the exact mechanism(s) underlying this regulation is poorly understood.

In a work performed by Calamita *et al.,* (2001), pH-dependent fluorophores and pH-dependent chemical modification reactions were used to show that the CW of respiring *B. subtilis* cells is protonated, having thus a relatively low pH environment. It has been proposed that this pmf-dependent acidification of the CW during growth could downregulate the activity of autolysins (Calamita *et al*., 2001; Kemper *et al*., 1993).

As already indicated, the majority of Gram-positive bacteria contain TAs, which are polyanionic glycopolymers associated to the CW. In *B. subtilis* and *S. aureus* proton and cation homeostasis in the CW during respiratory metabolism seems to depend on the presence of TAs (Calamita *et al*., 2001; Schirner *et al*., 2009; Biwas *et al*., 2012). Rice and Bayles (2008) proposed that the acidic pH could suppress the activity of autolysins associated with TAs by promoting the protonation of the D-Ala ester linkages of these CW polymers. Upon dissipation of the membrane potential, the pH in the cell wall increases and destabilizes or deprotonates the D-Ala ester linkages, activating autolysins (Rice and Bayles, 2008). Staphylococcal cell separation depends largely on the bifunctional autolysin Atl, which displays amidase and glucosaminidase activities (Heilmann *et al.*, 1997). Schlag *et al*., (2010) demonstrated that WTA prevented Atl binding to the PG in the bacterial side walls but not in the septum, where WTA appeared to be less abundant or have an altered structure. Similar observations have been made with the autolysins LytF of *B. subtilis* (Yamamoto *et al*., 2008) and Sle1 (or Aaa) and LytN of *S. aureus* (Frankel and Schneewind, 2012), all of which have motifs of the LysM family as CWBD. A recent study further supported the interrelationship between activity of the respiratory chain (a major source of protons in the CW), the presence of WTA and autolysis regulation, with authors proposing that WTA contribute to the control of autolysin activity by governing the abundance of protons in the CW (Biswas *et al*., 2012).

In spite of all the indicated studies, the mechanisms by which loss of the pmf induces autolysis are still largely unknown. It appears though that collapse of the CM proton gradient might activate enzymes with distinct substrate specificities or localization, somehow connected to the presence or absence of WTA and LTA (and their substituents) in the bacterial CW.

1.5 Exploration of endolysins as antibacterial agents

After years of almost exclusive use of antibiotics in the treatment of infectious diseases caused by bacteria, the emergence of multidrug resistant bacteria and the slowdown in the discovery of new classes of antibiotics (Wenzel, 2004; Theuretzbacher, 2012; Cole, 2014) has created the need to develop new strategies that ensure the elimination of these pathogens (Czaplewski *et al*., 2016). Phage endolysins by having the ability to degrade an essential component of the bacterial CW, the PG, have been intensively explored as alternative antimicrobial agents (for reviews see Nelson *et al*., 2012; Schmelcher *et al*., 2012a; Pastagia *et al*., 2013).

Many studies have shown that when applied externally, as purified recombinant proteins, endolysins can lead to 'lysis from without' of target bacteria (Borysowski *et al*., 2006; O'Flaherty *et al*., 2009; Fischetti, 2010). This effect is most obviously seen with Gram-positive bacteria, as the OM of Gram-negative and mycobacteria hinders endolysin access to the PG. Nevertheless, in recent years considerable progress has been made in the search of endolysins with killing activity against Gram-negative bacteria. Researchers are working on identifying naturally occurring endolysins capable of lysing these bacteria from the outside, on engineering endolysins with motifs for targeting and crossing of the OM, and on combining endolysins with OM-permeabilizing agents (Lai *et al*., 2011; Lukacik *et al*., 2012; Briers and Lavigne, 2015). The majority of the studied endolysins have a reduced lytic spectrum, which is tipically restricted to the species (or subspecies) of bacteria against which they were naturally designed to act on. There are some cases however of enzymes with broader lytic activity, as for example the enterococcal endolysin PlyV12 that can kill enterococci and other Gram-positive pathogens such as *S. agalactiae* and *S. pyogenes*, making it one of the broadest-acting endolysin ever identified (Yoong *et al*., 2004).

In 2001, Nelson *et al*., were the first to prove that an endolysin (PlyC from the streptococcal phage C1) could be used *in vivo* to protect mice from colonization of *S. pyogenes* in the upper respiratory tract. This and other results (Nelson *et al*., 2006) motivated the term enzybiotics, which is used to describe the therapeutic potential of phage endolysins. Endolysins have been evaluated in animal models of pneumoniae, endocarditis and sepsis with a focus on efficacy and host immune response (Loeffler *et*

al., 2001, 2003; Entenza *et al*., 2005; McCullers *et al*., 2007; Grandgirard *et al*., 2008; Witzenrath *et al*., 2009). Some reports highlighted the ability of endolysins to eliminate *S. aureus* biofilms, namely the endolysin of phage ϕ11 (Sass and Bierbaum, 2007), LysK (O'Flaherty *et al*., 2005), LysH5 (Gutiérrez *et al*., 2014) and SAL-2 (Son *et al*., 2010). A synergistic lytic effect has been described when two different endolysins (with distinct CDs) are simultaneously used, as observed for the pneumococcal endolysins Pal and Cpl-1 (Loeffler and Fishetti, 2003), or when endolysins are combined with other PG hydrolases (e.g. LysK in conjunction with lysostaphin, Becker *et al*., 2008). Endolysins and conventional antibiotics can also exhibit synergistic interaction, as demonstrated for Cpl-1 with either penicillin or gentamicin (Djurkovic *et al*., 2005).

The high affinity for bacterial CW substrates allows endolysins to quickly identify and kill target bacteria. Some of these lytic enzymes proved effective *in vivo*, either by protecting/rescuing mice after animal inoculation with pathogenic bacteria, or by promoting the decolonization of mucosal surfaces (Loeffler *et al*., 2001, 2003; Schuch *et al*., 2002; Daniel *et al*., 2010). This feature could also be important on disinfection of implanted medical devices, such as implantable cardiac devices, cardiac valves, orthopedic prostheses and indwelling catheters. Nevertheless, until now only PlyC has been tested specifically as an environmental disinfectant (Hoopes *et al*., 2009).

1.5.1 Engineering of endolysins

Endolysins exhibit important characteristics as antibacterial agents, but they can also present some limitations, such as narrow host range, low solubility during large scale production and reduced activity in *in vivo* experiments. During the last years, many examples of engineered phage endolysins have been reported that aimed to overcome these problems (see reviews Nelson *et al*., 2012; Schmelcher and Loessner, 2016). An interesting approach is the development of chimerical endolysins by swapping and/or combining different PG hydrolase domains, namely CDs and CWBDs (Yang *et al*., 2014). Some of these chimeras are listed in Table 1.1.

Table 1.1. Chimerical Endolysins and Main Features.

Chimerical Endolysin	Relevant Characteristics	Reference
	Fusion of PG Domains between Autolysins and Endolysins	
CLC muramidase	Acquired hydrolyzing activity against choline-containing pneumococcal CW.	Croux *et al*., 1993
λSA2-E-Lyso-SH3B	Activity against staphylococcal and streptococcal bovine mastitis isolates; synergy with lysostaphin.	Schmelcher *et al*., 2012b
PL_3	Better lytic performance than parental enzymes against *S. pyogenes* and other choline-containing bacteria (encapsulated and multiresistant isolates); improved stability; bactericidal activity *in vivo*.	Blázquez *et al*., 2016
	Fusion of PG Domains between Endolysins	
Ply187AN-KSH3b	Active against multiple staphylococcal strains; diminished the progression of endophthalmitis in mice.	Singh *et al*., 2014
Ply187N-V12C	Broad activity spectrum across multiple genera, staphylococcal, streptococcal and enterococcal strains.	Dong *et al*., 2015
PlyGVE2CpCWB	Active against *Clostridium perfringens*; improved thermostability.	Swift *et al*., 2015
Cpl-711	Improved lytic activity of the parental endolysins against *S. pneumoniaee in vivo* and *in vitro*.	Díez-Martínez *et al*., 2015
	Fusion between PG Domains of Phage Structural Proteins and Endolysins	
P16-17	Strong activity against *S. aureus*; improved solubility of the chimera over the parental enzymes; synergistic effect with gentamicin.	Manoharadas *et al*., 2009
EC300	Higher lytic activity than the parental endolysin Lys170 against *E. faecalis*, especially in growth-promoting media.	Proença *et al*., 2015a
	Duplication of PG Domains	
CBD500	Increase of CW binding affinity by approximately 50-fold in *Listeria* strains; great lytic performance at higher ionic strength.	Schmelcher *et al*., 2011

Likewise, engineered truncations of endolysins can yield enzymes with greater lytic specific activity and/or extended lytic spectrum when compared to the parental full-length PG hydrolases. This was verified with the staphylococcal and clostridial endolysins LysK and CD27L, respectively (Horgan *et al*., 2009; Mayer *et al*., 2011). Other truncations resulting in hyperactivity were reported for the streptococcal endolysin PlyGBS after application of random mutagenesis (Cheng and Fischetti, 2007).

As endolysins are below the 60-65 kDa cutoff, they are expected to be rapidly eliminated from the human body by glomerular filtration (Maack *et al*. 1979). Interestingly, the artificial dimerization of the pneumococcal endolysin Cpl-1 resulted in higher lytic activity and increased half-life in mice when compared to the natural monomeric form (Resch *et al*., 2011).

Regarding the utilization of endolysins against Gram-negative bacteria, different strategies have been pursued to overcome the physical barrier imposed by the OM, namely with endolysins targeting *Pseudomonas, E. coli* and *Salmonella* (Briers *et al*., 2011). For instance, endolysins K2144 and EL188 were able to display lytic activity against a broad range of Gram-negative bacteria when the OM was permeabilized with EDTA (Briers *et al*., 2007). Also, Artilysins, which are endolysins modified with LPS-destabilizing peptides, such as (poly)cationic peptides, presented interesting lytic effects against *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Briers *et al*., 2014). In another study, the T4 lysozyme was fused to the pesticin domain that is responsible for recognition and binding of this bacteriocin to the OM transporter FyuA. The fusion was shown to traverse the OM and kill *Yersinia* and pathogenic *E. coli* strains (Lukacik *et al*., 2012).

1.5.2 Enzibiotic therapy: aren't we missing something?

In spite of the wealth of reports pointing for the therapeutic potential of endolysins as antibacterial agents, it is important to perform a careful and critical analysis of these studies. Most endolysins and derived chimeras have only been tested *in vitro*, usually after washing and resuspension of target bacteria in nutrient-depleted, buffered solutions, which are unable to sustain normal pmf. As discussed above, these conditions generally make bacteria more prone to lysis and for some species they are even sufficient to induce autolysis. In fact, this could explain why the high lytic activity observed in these conditions sometimes does not translate to the expected results *in vivo*, in animal infection models. In most cases, satisfactory numbers of animal survival are only obtained when lytic enzymes are administrated to animals soon after the injection of the deadly bacterial inoculum, which generally is also prepared in a buffer solution (Loeffler *et al.*, 2003; Gu *et al.*, 2011; Oechslin *et al*., 2013). This experimental setting vaguely parallels what happens in real scenarios of infection. A study with a staphylococcal phage enzyme with

high lethality against MRSA, both *in vitro* and in a mouse model, showed that the enzyme could be used to decolonize staphylococci from the nares of mice as well as to protect the animals from an intraperitoneal challenge with MRSA. However, in the latter experiment, the best protection was observed if the endolysin was added up to 30 minutes after the MRSA challenge (Rashel *et al*. 2007). As already pointed out, better results can be achieved when endolysins are used in conjugation with other antimicrobial agents, such as the lantibiotic nisin (Garcia *et al*., 2010), penicillin and glycopeptide antibiotics (Rashel *et al*. 2007; Djurkovic *et al*., 2005).

It is important to note that endolysins are naturally designed to act from the cell inside and always after bacterial death caused by the holin pmf-dissipating action. In fact, it has been estimated that less than one thousand endolysin molecules per cell are sufficient to lyse phage-infected bacteria in a few seconds or minutes (Young and Wang, 2006). In a typical *in vitro* setting with 10^8 to 10^9 infected cells/ml this would correspond to an endolysin concentration clearly below the μg/ml scale (sometimes mg/ml) that is usually used to achieve lysis from the outside. Having this in mind, it is interesting to note that the fusion of a CWBD of a c-endolysin (Lys170) to a CD of a VAL resulted in a drastic improvement of bacterial cell lysis when compared with the parental endolysin Lys170 (Proença *et al*., 2015a) (Table 1.1). Unlike endolysins, VALs, as structural proteins of the phage virus particle, are naturally designed to act from without in actively growing bacteria.

Thus, the obvious question is why are bacterial cells apparently more resistant to exogenously-added endolysins when compared to their action in the phage-infection context, in which they reach the CW from inside. Although certainly the PG substrate facing the extracellular media exhibits differences in composition, ionic environment and accessibility relatively to the PG facing the CM, it is also possible that the holin pmf-dissipating action may be important to efficiently sensitize cells to all endolysins, and not just to e-endolysins (see next chapters).

1.6 Thesis goals

The main goal of this doctoral project was to contribute to the understanding of the mechanisms and factors that influence the lytic performance of phage endolysins, and how they might impact the current exploitation of these enzymes as antibacterial agents.

In the phage infection context all known endolysins act after holin-mediated cell death. Enzybiotic therapy envisages the exogenous application of recombinant forms of these enzymes to lyse viable bacterial cells. Previous work showing reduced solubility and poor lytic activity of different recombinant endolysins has motivated the first goal of this work, that is, the production of chimeric endolysins active against clinically relevant strains of *S. aureus* (Chapter 2).

Despite the interesting results obtained with the chimeras, we still observed that these and other endolysins failed to efficiently lyse bacteria when growing actively in rich media. This fact, combined with the previous findings on e-endolysins, led us to hypothesize that the holin-pmf dissipating function could be a general factor that guarantees full activity of endolysins (c- and e-endolysins). Taking advantage of the accumulated knowledge on the molecular biology of *B. subtilis* and its phage SPP1, and profiting from the availability of molecular tools for their genetic manipulation, we have selected this bacterium/phage system as a model to test our hypothesis. To that end we have addressed the following essential questions (Chapter 3): can fully energized bacterial cells naturally counteract the activity of exogenously added c-endolysins? Can this counteraction be abolished in presence of drugs that dissipate the pmf? ii) Is the holin-mediated dissipation of the pmf required to render cells fully susceptible to c-endolysins? With the knowledge and tools that emerged from this study we extended our goals to perform a functional characterization of the phage SPP1 holin (Chapter 4).

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CHAPTER 2.

NOVEL CHIMERICAL ENDOLYSINS WITH BROAD ANTIMICROBIAL ACTIVITY AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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Novel Chimerical Endolysins with Broad Antimicrobial Activity against Methicillin-Resistant *Staphylococcus aureus*

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Author's Note

In the Discussion of the following chapter it is highlighted the striking similarity between the enterococcal phage endolysin Lys168 and a previously described endolysin from phage SAP6. In a first registry, this phage was assigned in sequence databases as being from *S. aureus*. However, soon after the approval for publication of the work presented in this chapter, the SAP6 genome and endolysin entries in databases, GenBank JF731128 and AEM24735, respectively, were corrected and it turned out that SAP6 appears also to be an *E. faecalis* phage. This should be taken into account when reading the following chapter.

Author Disclosure Statement

Technophage has proprietary rights over *E. faecalis* phages F168/08, F170/08 and *S. aureus* phage F87s/06, their encoded endolysins Lys168, Lys170 and Lys87, and the derived chimerical enzymes Lys168-87 and Lys170-87. All authors reviewed and approved the present manuscript before submission. All authors declare that there are no conflicts of interest. All authors contributed to the elaboration of the article. S.F., D.P. and C.C. were responsible for endolysin cloning, production and purification; SF executed all experiments evaluating lytic action of chimerical endolysins; F.A.S., C.L., C.C. and S.L. were responsible for phage isolation, purification and extraction of phage DNA; C.L. and S.L. performed phage genomic analysis; C.M. was responsible for handling and molecular typing of *S. aureus* typed clinical strains. H.deL., P.C.-S., M.G., M.P. and C.S.-J. were responsible for the experimental design and work supervising; C.S.-J. coordinated the collaborative work of the involved laboratories.

Abstract

Due to their bacterial lytic action, bacteriophage endolysins have recently gained great attention as a potential alternative to antibiotics in the combat of Gram-positive pathogenic bacteria, particularly those displaying multidrug resistance. However, large-scale production and purification of endolysins is frequently impaired due to their low solubility. In addition, a large number of endolysins appear to exhibit reduced lytic efficacy when compared with their action during phage infection. Here, we took advantage of the high solubility of two recently characterized enterococcal endolysins to construct chimeras targeting *Staphylococcus aureus*. The putative cell wall binding domain of these endolysins was substituted by that of a staphylococcal endolysin that showed poor solubility. Under appropriate conditions the resulting chimeras presented the high solubility of the parental enterococcal endolysins. In addition, they proved to be broadly active against a collection of the most relevant methicillin-resistant *S. aureus* epidemic clones and against other Gram-positive pathogens. Thus, fusion of endolysin domains of heterologous origin seems to be a suitable approach to design new potent endolysins with changed and/or extended lytic spectrum that are amenable to large-scale production.

2.1 Introduction

After decades of almost exclusive use of antibiotics in the treatment of infectious diseases caused by pathogenic bacteria, the emergence of multiresistant bacterial strains combined with a slowdown in the discovery of new classes of antibiotics is currently viewed as a major public health problem (Doyle *et al*., 2011; Willems *et al*., 2011; Woodford *et al*., 2011). Among the different bacterial pathogens that have acquired antibiotic resistance *Staphylococcus aureus* is still one of the most serious threats worldwide, being able to develop resistance to virtually all classes of antibiotics (de Lencastre *et al*., 2007). Methicillin-resistant *S. aureus* (MRSA) has been a leading cause of nosocomial infections for some decades and more recently has also emerged as a community-associated pathogen (Annual report, EARS-Net 2010; Chambers, 2001, Furuya and Lowy, 2006, Klevens *et al*., 2007). *S. aureus* is responsible for a wide number of infectious diseases ranging from skin and soft tissues to fatal bloodstream infections, endocarditis, meningitis and bovine mastitis in dairy herds (Gruet *et al*., 2001; Larkin *et al*., 2007). For all these reasons there is an urgent need to develop new antibacterials that ensure elimination of multidrug-resistant pathogenic bacteria, particularly MRSA strains.

Among the different approaches to obtain new antibacterials one that has recently caught great attention is the exploitation of bacteriophage endolysins (Fenton *et al*., 2010; Fischetti, 2010; Loessner, 2005; O'Flaherty *et al*., 2009). Endolysins are bacterial cell wall hydrolyzing enzymes that promote host cell lysis in the end of the lytic cycle of double-stranded DNA phages, thus allowing efficient release of the viral progeny to the extracellular medium (São-José *et al*., 2007). Despite the fact that endolysins have been evolutionarily designed to operate from the inside of infected cells, when applied exogenously as purified recombinant proteins they have been shown to promote lysis of different Gram-positive pathogenic bacteria, including *S. aureus* (Loeffler *et al*., 2001; Nelson *et al*., 2001; O´Flaherty *et al*., 2005; Yoong *et al*., 2004).

Most endolysins have a modular organization with a conserved N-terminal catalytic domain (CD) and a more diverse C-terminal cell wall binding domain (CWBD) (Fischetti, 2008; Garcia *et al*., 1990). However, several *S. aureus* phages produce endolysins with two catalytic domains in their N-terminus, such as those from phages K, ϕ11 and ϕMR11. These lytic enzymes present a CHAP (cysteine, histidine-dependent

amidohydrolases/peptidases) domain followed by an Amidase-2 domain (*N*-acetylmuramoyl-L-alanine amidase), where the CHAP seems to be the most effective in inducing lysis (Navarre *et al*., 1999; O´Flaherty *et al*., 2005; Raschel *et al*., 2007). Other feature commonly reported for *S. aureus* phage endolysins is their poor solubility when overexpressed in *Escherichia coli*, frequently leading to low yields of soluble protein (Becker *et al*., 2008; Garcia *et al*., 2010; Manoharadas *et al*., 2009; O´Flaherty *et al*., 2005).

We have experienced this insolubility problem when attempting to purify a broad-host-range anti-staphylococcal endolysin available in our lab, Lys87 (Cantante, 2008). Here, we describe a new strategy to overcome this problem that consisted in the construction of new chimerical endolysins composed of the Lys87 CWBD fused to the highly soluble CDs of two *Enterococcus faecalis* phage endolysins, Lys168 and Lys170 (Proença *et al*., 2012). By employing this strategy, complemented with optimized expression conditions, we obtained two highly soluble lytic enzymes that are broadly active against a large cohort of *S. aureus* strains, including MRSA, as well as against other staphylococcal, streptococcal and enterococcal species. To our knowledge, this corresponds to the first report of chimeras comprising domains of *S. aureus* and *E. faecalis* phage endolysins.

2.2 Results

2.2.1 Production of chimerical endolysins targeting *S. aureus*

We have recently characterized three phage endolysins, two from *E. faecalis* phages F170/08 and F168/08 and another from *S. aureus* phage F87s/06 (Cantante, 2008; Proença *et al*., 2012). *In silico* analysis of the endolysin from phage F170/08, that is, Lys170 identified a CD of the Amidase-2 family in its amino terminal region (Figs 2.1A and S2.21A). This family includes zinc amidases that have N-acetylmuramoyl-L-alanine amidase activity (Cheng *et al*., 1994). The same analysis performed with endolysins Lys168 and Lys87 from phages F168/08 and F87s/06, respectively, revealed a CD of the CHAP family (Bateman and Rawlings, 2003; Layec *et al*., 2008; Rigden *et al*., 2003), also located in the enzymes' N-terminal region (Figs 2.1A and S2.1A). Enzymes from this family have been shown to cleave different amide bonds in the peptidoglycan mesh, frequently displaying amidase or endopeptidase activity.

Lys170 and Lys168 could be easily produced in *E. coli* in its soluble form whereas Lys87 showed a high propensity to precipitate as inclusion bodies in all tested conditions (Cantante, 2008; Proença *et al*., 2012). The enterococcal endolysins exhibited a narrow lytic spectrum with their activity being basically restricted to *E. faecalis* cells (Proença *et al*., 2012). This feature might result from CWBD binding to a specific epitope present on the cell wall of *E. faecalis* cells and/or dependence of the CD on CWBD binding for activity (Low *et al*., 2011). Lys170 showed better lytic performance when both enterococcal endolysins were tested against a large number of *E. faecalis* clinical strains (Proença *et al*., 2012).

Having this in mind we have envisaged that replacing the CWBD of Lys170 and Lys168 by that of Lys87 would result in chimerical endolysins (Figs 2.1A and S2.1B) maintaining the high solubility properties of the parental enterococcal enzymes and the ability to recognize and act on *S. aureus* cells conferred by the parental Lys87 CWBD.

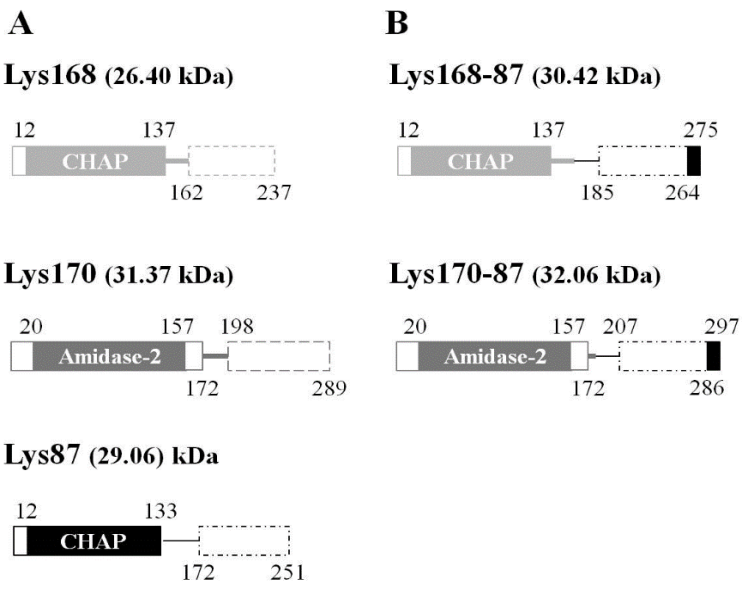

Fig. 2.1. Schematic representation of parental (A) and resulting chimerical endolysins (B). N-terminal conserved domains (CD) are depicted as filled rectangles with indication of functional families (CHAP and Amidase-2). Dashed rectangles delimit the C-terminal region that must contain the putative cell-wall binding domain (CWBD). Linker regions connecting CD and CWBD are indicated by a line with the filling code and thickness of parental endolysins. The black rectangle at the end of each chimera represents the C-terminal hexahistidine tag used for affinity chromatography purification. The amino acidic coordinates of functional and linker domains as well as the total length of each endolysin are indicated above and below the schemes.

The coding sequences of the functional domains composing each chimerical endolysin were PCR-amplified, fused by OE-PCR and cloned in the *E. coli* expression vector pIVEX2.3d, which allowed production of the chimeras C-terminally fused to a hexahistidine tag (Figs 2.1B, S2.1B and Materials and methods). The ability of the chimerical enzymes Lys168-87 and Lys170-87 to lyse *S. aureus* cells was initially confirmed by growing the resulting protein-expressing *E. coli* clones on a dense lawn of autoclavated staphylococcal cells and checking for the presence of lysis halos around the *E. coli* colonies (Fig. S2.2).

By employing standard protein expression conditions (37ºC, 3-4 h production after culture induction) with selected clones we could obtain much higher soluble amounts of the chimerical endolysins than those we had obtained previously with Lys87. However, the quantity of soluble Lys168-87 and Lys170-87 did not match the levels we achieved with the parental enterococcal endolysins as we anticipated (not shown). This could be overcome though by simply incubating the induced cultures at 16ºC during 14 h. In these conditions we achieved similar or higher soluble quantities of the chimeras when compared to the native enterococcal enzymes. The chimeras were subsequently purified from total soluble extracts by affinity chromatography using nickel columns. Pure fractions were pooled and subjected to a desalting step for protein exchange to an imidazole-free, sodium phosphate-based buffer (Fig. 2.2). Average yields were 20 and 30 μg of pure Lys168-87 and Lys170-87, respectively, per ml of induced culture.

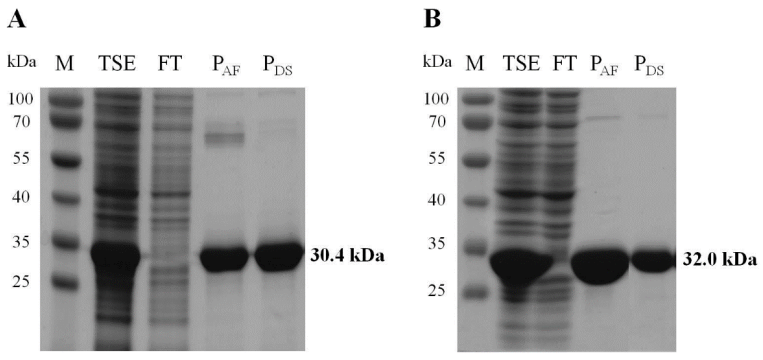

Fig. 2.2. SDS-PAGE analysis of Lys168-87 (A) and Lys170-87 (B) purification. M, molecular weight marker; TSE, total soluble extract loaded in the affinity column; FT, flowthrough of the affinity column; P_{AF}, eluted fraction of the affinity peak; P_{DS}, eluted fraction of the desalting peak.

2.2.2 Lytic action of the chimerical endolysins against clinical *S. aureus*

The lytic action of Lys168-87 and Lys170-87 against *S. aureus* was firstly assessed using a panel of 100 clinical isolates from Technophage's collection (Table S2.1), which were isolated from different infection contexts and obtained both from Portuguese community and hospital settings. This panel included 42 isolates identified as MRSA. The lytic capacity of each chimera was evaluated by spotting four different amounts of pure enzyme (10, 5, 1 and 0.2 μg) on a dense lawn of viable cells from each tested isolate, which was produced by incorporating cells from exponentially growing cultures in a soft-agar, phosphate-buffered medium (see Material and methods). The chimera-induced lysis was qualitatively evaluated by scoring the relative diameter and turbidity of the lysis halos produced after overnight incubation at 37ºC (Table S2.2).

For the highest tested protein amount (10 μg) we observed that both chimeras were able to produce lysis halos in more than 90% of the isolates (Fig. S2.3). For each protein quantity though, Lys168-87 generally produced larger and more transparent lysis halos than Lys170-87 (Table S2.2). The narrowing of the lytic spectrum of this chimera as the spotted protein amount decreased was more pronounced than that observed with Lys168-87 (Fig. S2.3). Interestingly, these results were basically opposite to those we obtained with the parental Lys168 and Lys170, where the later exhibited better lytic performance against *E. faecalis* (Proença *et al*., 2012).

Although both chimerical endolysins revealed to be very effective in lysing this panel of *S. aureus* clinical isolates (n = 100), these were not typed and therefore the diversity present in this group was unknown. In order to have a more precise view on the lytic potential of the chimeras against *S. aureus*, the enzymes were similarly tested in a panel composed of diverse and typed MRSA and MSSA strains (Table 2.1). This strain collection was previously characterized in terms of their genetic background and included representatives of the most relevant MRSA pandemic clones from different parts of the world and representatives of the dominant of MSSA clones.

Table 2.1. Detailed Description of Typed *S. aureus* Clinical Strains

Strain ID	Origin	Isolation date	spa typing Ridom	MLST (ST)	CC[1]	SCC mec type	Reference[2]
							MRSA, n = 30
N315	Japan	1982	t002	5	5	II	15, 18
USA100	USA	1995-2003	t002	5	5	II	16
JP1	Japan	1987	t002	5	5	II	3, 18
BM18	USA	1989	t002	5	5	IVa	6, 18
HDE288	Portugal	1996	t311	5	5	VI	18, 21
COB3	Colombia	1996	t1107	5	5	IV_{NT}	10, 18
USA300	USA	1995-2003	t008	8	5	IVa	5
GRE120	Greece	1993	t036	8	5	IVh	1
JH1	USA	2000	t002	105	5	II	24
JH9	USA	2000	t002	105	5	II	24
ANS46	Australia	1982	t037	239	5	III	8,12,18
DEN907	Denmark	2001	t037	239	5	NT	9, 12
HDG2	Portugal	1992	t421	239	5	IIIB	12, 23
HPV107	Portugal	1992	t051	247	5	IA	18, 22
BK1953	USA	1995	t051	247	5	IA	18, 19
PER184	Spain	1991	t121	247	5	IA	7, 18
BK793	Egypt	1961	t008	250	5	I	14, 18
COL	UK	1965	t051	250	5	I	18
MW2	USA	1998	t128	1	15	IVa	4
USA400	USA	1995-2003	t558	1	15	IVa	16
HAR22	Finland	2002	t032	22	22	IVh	11, 20
HGSA146	Portugal	2003	t032	22	22	IVh	1
DEN2294	Denmark	2001	t019	30	30	IVc	9
DEN2946	Denmark	2001	t975	30	30	IVc	9
USA200	USA	1995-2003	t018	36	30	II	16
HAR24	Finland	2002	t018	36	30	II	11, 20
HAR38	Belgium	1995	t004	45	45	IVa	11, 20
WIS	Australia	1995	t123	45	45	V	13
GRE14	Greece	1998	t044	80	80	IV	1
DEN114	Denmark	2001	t044	80	80	IVc	9
							MSSA, n = 13
HSA29	Portugal	1992-1993	t002	5	5	NA	2
NCTC8325	UK	1943	t211	8	5	NA	5, 17
DCC403	Portugal	1996-1997	t148	615	5	NA	2

Table 2.1 (continued)

Draftees728	Portugal	1996-1997	t213	12	12	NA	2
IPOP33	Portugal	2001	t1071	9	15	NA	2
DCC192	Portugal	1996-1997	t084	15	15	NA	2
DCC750	Portugal	1996-1997	t078	25	15	NA	2
IPOP58	Portugal	2001	t189	188	15	NA	2
Draftees721	Portugal	1996-1997	t790	22	22	NA	2
DCC1060	Portugal	1996-1997	t012	30	30	NA	2
IPOP34	Portugal	2001	t884	34	30	NA	2
DCC457	Portugal	1996-1997	t330	45	45	NA	2
IPOP74	Portugal	2001	t645	121	121	NA	2

[1]Clonal complexes (CC) were determined using the E-burst software http://saureus.mist.net/, last accessed on January 25, 2012. [2]The references corresponding to the numbers indicated in the Table can be found in section 2.6.1. Abbreviations: NA, not applied; NT, non-typeable; MLST (ST), Multilocus Sequence Typing (Sequence Type); SCC*mec*, Staphylococcal Chromosomal Cassette *mec*

Remarkably, the chimerical endolysins lysed between 70 to 100% of this panel of typed *S. aureus* clinical strains, depending on the amount of tested enzyme (Fig. 2.3). Lys168-87 showed again a better lytic performance when compared to Lys170-87 since 0.2 μg of the former were sufficient to produce a lysis halo in 83% of the tested MRSA strains, whereas Lys170-87 lysed only 70%. A detailed description of the lytic activity of the endolysins in each particular *S. aureus* strain is shown in Table S2.3.

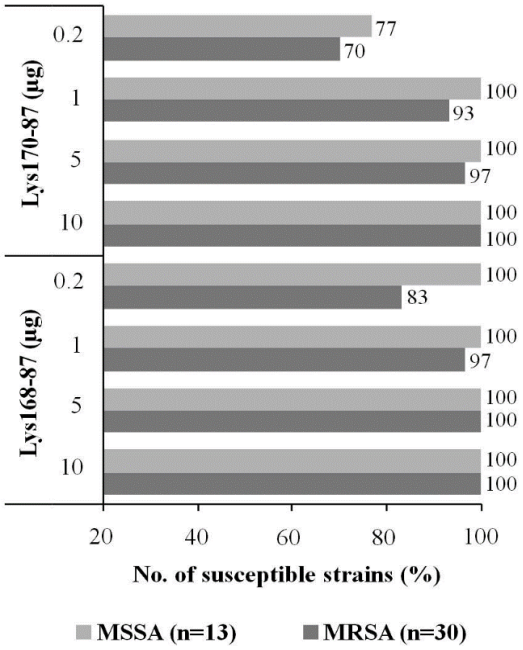

Fig. 2.3. Susceptibility of a panel of diverse and typed *S. aureus* clinical strains to the lytic action of Lys168-87 and Lys170-87. Typed MRSA and MSSA strains (Table 2.1) were challenged by the spot assay (see text) with the indicated amounts of each chimera. The percentage of strains that developed a lysis halo after overnight incubation at 37ºC is plotted as a function of each enzyme amount. A detailed description of the susceptibility of each strain to both chimeras is presented in Table S2.3.

2.2.3 Lys168-87 and Lys170-87 have a synergistic lytic effect in liquid media

The chimerical endolysins were also able to induce lysis of viable *S. aureus* cells in dense liquid suspensions, as shown in the example of Fig. 2.4 for the MRSA epidemic clone USA200. When applied separately, each endolysin caused a drop of cell suspension turbidity to about 40% of the initial value, after 1 h incubation, which translated into a corresponding decrease of the initial CFU/ml. However, if simultaneously added to cultures, each at half of the concentration when used separately, the chimerical enzymes decreased the cell suspension turbidity to almost 5% of the initial value and eliminated ca. 99% of the initial CFU/ml (Fig. 2.4). These results indicate a synergistic effect of both endolysins in the effective lysis and killing of *S. aureus*.

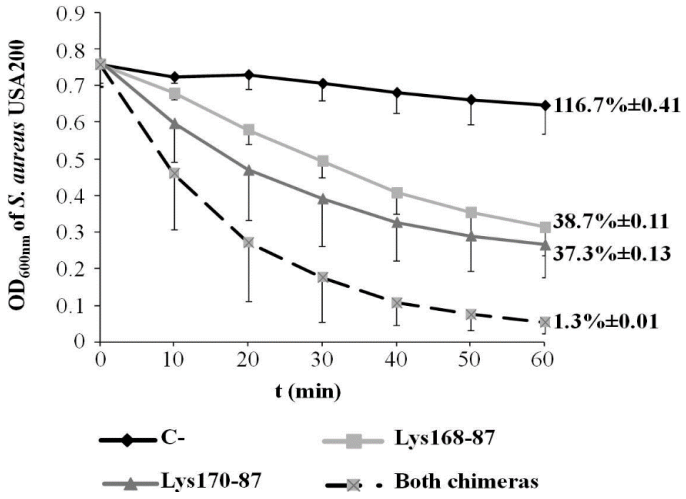

Fig. 2.4. Lytic action of Lys168-87 and Lys170-87 against *S. aureus* strain USA200 in liquid media. The graphic shows the change of cell suspensions OD_{600} over time, after adding each chimera separately (10 μg/ml) or in combination (5 μg/ml each). Endolysin buffer was added to the negative control (C-) instead of the enzymes. Values are the means of three independent experiments with indication of standard deviation. The values on the right side indicate the percentage of the initial CFU/ml after 60 min of enzymes' action and the corresponding standard deviation.

2.2.4 Activity of the chimerical endolysins against other Gram-positive pathogenic bacteria

As mentioned before, the parental enterococcal endolysins Lys168 and Lys170 showed a clear preference for *E. faecalis* when tested against a representative group of typed strains of this species and of *Enterococcus faecium*. When comparing their lytic action, Lys170 showed to be more effective than Lys168 (Proença *et al.*, 2012). By replacing their putative CWBD by that of the staphylococcal endolysin Lys87 we could efficiently retarget their activity towards *S. aureus*, as initially envisaged (see above). Next we studied how the chimeras acted on Gram-positive pathogenic bacteria other than *S. aureus* (Table S 2.4). When tested against the indicated *E. faecalis* and *E. faecium* clinical isolates, the chimerical enzymes essentially reproduced the lytic profile displayed by the parental enterococcal endolysins (Proença *et al.*, 2012), with Lys170-87 producing larger and clearer lysis halos compared to Lys168-87 (Fig. 2.5 and Table S 2.5). Note that the *E. faecalis* and *E. faecium* isolates tested here were not genetically characterized and the

vast majority of them have been shown previously to be susceptible to both Lys168 and Lys170 (Proença *et al*., 2012).

Interestingly, and in contrast to the parental enterococcal endolysins, the chimeras were also able to cause lysis of clinical isolates of other staphylococcal pathogens and Group A streptococci (Fig. 2.5A and Table S2.5). Qualitatively, Lys168-87 demonstrated equal or superior lytic action than Lys170-87 against all tested bacteria, except for the enterococcal isolates where Lys170-87 showed to be more powerful (Fig. 2.5B and Table S2.5).

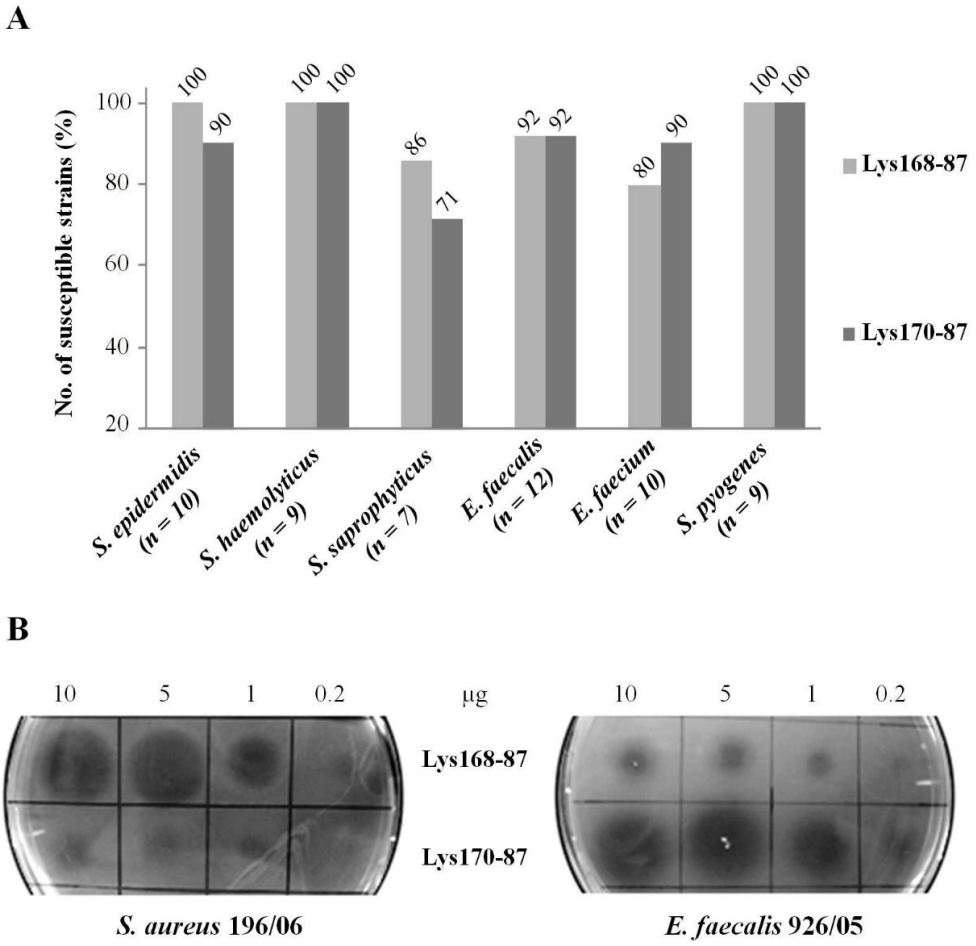

Fig. 2.5. Lytic action of Lys168-87 and Lys170-87 in other Gram-positive bacterial pathogens. (**A**) Bar plot showing the percentage of isolates from each species (Table S2.4) that developed a lysis halo after spotting 10 µg of each chimera. A detailed description of the susceptibility of each isolate to this and lower quantities of each chimera is presented in Table S2.5. (**B**) Representative lysis halos for the indicated amounts of each chimera in a dense lawn of viable cells from *S. aureus* and *E. faecalis* isolates 196/06 and 926/05, respectively.

2.3 Discussion

A survey of the literature indicates that researchers frequently have to deal with the poor solubility of different endolysins, including those of *S. aureus* phages, when aiming their production and purification (Becker *et al*., 2008; Garcia *et al*., 2010; Manoharadas *et al*., 2009; O´Flaherty *et al*., 2005; Schmitz *et al*., 2011; Yoong *et al*., 2004). This is also a recurrent problem in our lab and generally the available technical approaches to increase solubility, or to refold proteins recovered from inclusions bodies, are time consuming and poorly effective.

We propose here that construction of chimeras, where we take advantage of the heterologous, highly soluble endolysin functional domains, may represent a solution to obtain large amounts of active lytic enzymes with retargeted and/or extended lytic spectrum relatively to parental endolysins. As far as we know, this is the first time that such approach is employed with the specific objective of obtaining highly soluble lytic enzymes targeting *S. aureus*. There is though at least one report where a similar strategy was attempted to solve the insolubility problem of an *S. aureus* phage endolysin. In this case the putative CD of the endolysin P16 from phage P68 was fused to the inferred CWBD of the virion associated lysin P17, also from the same phage. However, the resulting chimera remained insoluble and accumulated as inclusion bodies (Manoharadas *et al*., 2009).

The construction of chimerical enzymes as a mean to improve the lytic efficacy or change the lytic spectrum, both *in vitro* and *in vivo*, towards relevant pathogens has been previously described. Recently, a chimerical endolysin (ClyS) assembling a CD and a CWBD from different *S. aureus* phages, and in which the latter displayed *Staphylococcus*-specific binding, was shown to lyse different *S. aureus* MRSA and MSSA strains and several coagulase-negative *Staphylococcus* species (Daniel *et al*., 2010). This chimera revealed to be more effective than mupirocin for skin decolonization of MRSA and MSSA (Pastagia *et al*., 2011). In another example of homologous fusion, the CHAP domain of LysK, the endolysin from *S. aureus* phage K, was fused to the CWBD of the bacteriocin lysostaphin from *Staphylococcus simulans* (Idelevich *et al*., 2011). The recombinant endolysin PRF-119 exhibited antimicrobial activity against a

great number of MSSA and MRSA clinical isolates, with an average MIC_{90} of 0.391 μg/ml.

Heterologous fusion constructs where the native CWBD of the streptococcal LambdaSa2 endolysin Cpl-7 was replaced by that of either lysostaphin or LysK resulted in a ~5-fold increase in staphylolytic activity mediated by the Cpl-7 CD, while maintaining significant streptolytic activity (Becker *et al*., 2009). The results suggested that the CWBD of *S. aureus* lytic enzymes may not always be staphylococcal-specific. Yet in another example, chimeras composed of the streptococcal phage endolysin B30, or of its CHAP CD only, fused to the mature form of lysostaphin were shown to display lytic specificity for streptococcal pathogens and *S. aureus* (Donovan *et al*., 2006).

In the work reported here we have fused the CD of two highly soluble *E. faecalis* phage endolysins, Lys168 and Lys170, to the CWBD of the staphylococcal phage endolysin Lys87, which showed high propensity to precipitate in the form of inclusion bodies. Our rational was that the CD of the enterococcal enzymes would confer high solubility to the resulting chimerical endolysins. In addition, the fact that Lys168 and Lys170 CDs were of the CHAP and Amidase-2 families, respectively, increased the chances of these domains to act on the *S. aureus* cell wall, if targeted by the Lys87 CWBD. Actually, the Lys168 CHAP domain, as deduced by Pfam analysis, shares 98% identity with that of the endolysin of *S. aureus* phage SAP6 (Proença *et al*., 2012). Lys170 Amidase-2 CD should confer N-acetylmuramoyl-L-alanine amidase activity as this was the activity experimentally determined for ORF9, an endolysin which is virtually identical to Lys170 (Proença *et al*., 2012; Uchiyama *et al*., 2011). Amidases cleave the amide bond that links the N-acetyl muramic acid of glycan strands to the L-alanine residue of peptide stems. This bond and linked residues are common to the vast majority of bacterial cell wall peptidoglycans, including that of *S. aureus* (Schleifer *et al*., 1972).

The chimeras thus constructed proved to efficiently lyse *S. aureus* clinical strains. When tested at their highest concentration each recombinant endolysin was able to induce lysis in more than 96% of 143 *S. aureus* clinical isolates. The lytic efficiency of each chimera varied among the different isolates, as judged by the relative size and transparency of the lysis halos (Tables S2.2 and S2.3). Remarkably, all the 30 typed strains composing a panel representative of the most relevant MRSA epidemic clones (Table 2.1) showed to be

susceptible to both chimerical enzymes. Very few anti-staphylococcal endolysins have been tested in such a representative group of MRSA typed strains.

Also interesting was the fact that these chimerical endolysins not only essentially maintained the lytic performance of the parental enterococcal endolysins when tested against *Enterococcus* (Proença *et al*., 2012), but were also extended in their lytic spectrum being active against other *Staphylococcus* species and *S. pyogenes*. This suggests that the Lys87 CWBD may target a bacterial cell wall epitope that is common to several Gram-positive bacterial species, while that of the parental enterococcal endolysins binds to a ligand predominantly found on enterococcal cell wall. This apparent wider target range of the CWBD of anti-staphylococcal lytic enzymes was also observed for lysostaphin and LysK, as mentioned above. Thus, Lys168-87 and Lys170-87 can be added to the small list of broadly active, phage-derived lytic enzymes (Becker *et al*., 2009; Donovan *et al*., 2006; Yoong *et al*., 2004).

A curious difference was observed when we compared the lytic efficiency of Lys168 and Lys170 with that of the derived chimeras Lys168-87 and Lys170-87. When tested against their natural target, *E. faecalis*, Lys170 generally produced larger and more transparent lysis halos than Lys168. However, this scenario was inverted when Lys168-87 and Lys170-87 were assayed against *S. aureus* (see illustrative example in Fig. 2.5B). This seems to indicate that the lytic performance of a given endolysin is not a mere function of the affinity and binding of the CWBD to the cell wall. In fact, other parameters such as the affinity of the CD to its substrate may influence lytic activity (Low *et al*., 2011).

Lys168-87 and Lys170-87 could also lyse liquid cell suspensions of viable *S. aureus*. In addition, they showed a clear synergistic effect when simultaneously added to cells (Fig. 2.4). To the best of our knowledge, such synergy between phage-derived hydrolytic enzymes in the effective lysis and killing of *S. aureus* has never been reported. Several studies though have demonstrated the occurrence of synergy between *S. aureus* phage endolysins and bacteriocins such as nisin (Garcia *et al*., 2010) or lysostaphin (Becker *et al*., 2008) or between endolysins and conventional antibiotics (Daniel *et al*., 2010; Djurkovic *et al*., 2005; Manoharadas *et al*., 2009; Rashel *et al*., 2007).

In conclusion, our results indicate that the engineering of chimerical endolysins from heterologous functional domains can be a good strategy to obtain large quantities of soluble and highly effective peptidoglycan hydrolases, either with narrow or broad lytic spectrum. The efficacy of the chimeras Lys168-87 and Lys170-87 is currently under study in animal models of *S. aureus* infection.

2.4 Materials and methods

2.4.1 Bacteria, culture media and growth conditions

The *E. coli* cloning strain XL1-Blue MRF' and its derivatives were grown at 37ºC with aeration in Luria-Bertani (LB) medium (Sambrook and Russel, 2001). The *E. coli* expression strain CG61 (São-José *et al*., 2000) and its derivatives were grown in LB in the same conditions, except that incubation temperature was 28ºC before induction of protein production and 16ºC afterwards. When appropriate, LB medium was supplemented with kanamycin (40 μg/ml) and/or ampicillin (100 μg/ml) for plasmid selection. Lytic action of chimerical endolysins was assayed in 200 bacteria clinical isolates (Tables 2.1, S2.1 and S2.4). Table 2.1 corresponds to a panel of 30 MRSA and 13 Methicillin-sensitive *S. aureus* (MSSA) typed strains. Table S2.1 lists 100 *S. aureus* isolates from Technophage's collection, 42 MRSA, 29 MSSA and 29 with unknown methicillin susceptibility, which were obtained from different Portuguese community and hospital settings between 2005 and 2008. Recombinant lytic enzymes were also tested in clinical isolates of other species, namely against *Staphylococcus epidermidis* (n = 10), *Staphylococcus haemolyticus* (n = 9), *Staphylococcus saprophyticus* (n = 7), *Enterococcus faecalis* (n = 12), *Enterococcus faecium* (n = 10), and *Streptococcus pyogenes* (n = 9) (Technophage collection, Table S2.4). The growth media for these bacteria were purchased from Biokar Diagnostics, Beauvais, France. *Staphylococcus* and *Enterococcus* species were cultured in Brain-Heart Infusion (BHI) and *S. pyogenes* in Todd-Hewitt broth supplemented with 2% of yeast extract (THY). Bacteria were grown at 37ºC, with aeration, except for *Enterococcus* that was grown without agitation. When necessary, 1.5% or 0.7% agar was added to these culture media in order to obtain solid or soft-agar plates, respectively. *E. faecalis* and *S. aureus* phages were propagated and purified by standard methods (Clokie and Kropinski, 2009; Kutter and Sulakvelidze,

2004), either in soft-agar media or liquid broth supplemented with $CaCl_2$ and $MgCl_2$ (5 mM each).

2.4.2 Identification and bioinformatics analysis of phage endolysins

Genomic DNA from *E. faecalis* and *S. aureus* phages was extracted from CsCl-purified lysates (Vinga *et al*., 2012) and their complete nucleotide sequence determined (service purchased to Macrogen, Seoul, Korea). DNA homology searches were carried out with BLASTN (Zhang *et al*., 2000), using the NCBI non-redundant nucleotide sequences database. Recognition of phage genome putative genes was performed by integrating the results obtained with GeneMark.hmm and MetaGeneAnnotator web software (Besemer and Borodovsky, 2005; Noguchi *et al*., 2008). Endolysins Lys168 and Lys170 from *E. faecalis* phages F168/08 and F170/08, respectively, were characterized previously (Proença *et al*., 2012). Identification of *S. aureus* phage F87s/06 endolysin gene was based on BLASTP homology searches (Altschul *et al*., 1997) against the NCBI non-redundant protein sequences database, using the deduced gene product, and on prediction of protein functional domains using NCBI's CDD (Marchler-Bauer *et al*., 2011) and Pfam (http://pfam.xfam.org/). Assignment of putative linkers connecting protein functional domains was performed with SVM (Ebina *et al*., 2009) and the SVM-joint output. Multiple protein sequence alignments were performed with ClustalW2 (Larkin *et al*., 2007).

2.4.3 Construction and cloning of *lys168-87* and *lys170-87* chimerical genes

The coding sequences of the N-terminal regions of the enterococcal phage endolysins Lys168 and Lys170, which included CHAP and Amidase-2 CDs, respectively (Proença *et al*., 2012) (Figs 2.1A and S2.1A), were PCR amplified from the corresponding phage DNA using a high fidelity *Pfu* DNA Polymerase (Fermentas Molecular Biology Tools, Thermo Scientific). The sequence encoding the C-terminal region of the staphylococcal phage endolysin Lys87 (Figs 2.1A and S2.1A), and which harbored the putative CWBD, was similarly amplified by PCR in a separate reaction. The forward primers used for CD amplification carried in their 5' end an N*co*I restriction site whereas the reverse primer employed in CWBD amplification contained an X*ma*I site. The reverse and forward primers used in CD and CWBD amplification, respectively, carried a 26-bp complementary segment in their 5' end. This allowed fusing the coding sequence of each

enterococcal endolysin CD to that of the CWBD of Lys87 by the technique of Overlap-Extension by Polymerase Chain Reaction (OE-PCR) (Ho *et al*., 1989), using the CD forward and CWBD reverse primers and a mixture of the initial PCR products as template. The resulting PCR products, corresponding to the chimerical genes *lys*168-87 and *lys*170-87 (Figs 2.1B and S2.1B), were purified using the *High Pure PCR Product Purification Kit* (Roche Applied Science), double-digested with N*co*I and *Xma*I and ligated to the equally digested pIVEX2.3d expression vector (Roche Applied Science). This vector is designed to drive the expression of cloned genes under the control of the phage T7 ϕ10 promoter and to allow the production of the corresponding proteins C-terminally fused to a hexahistidine tag. The *E. coli* strain XL1-Blue MRF' was transformed with these ligations as described previously (Chung *et al*., 1989). Transformants were selected in presence of 100 μg/ml ampicillin and screened for the presence of the desired recombinant plasmids by PCR using insert and vector complementary primers. Plasmid DNA from positive clones was extracted (*Pure Link Quick Plasmid Miniprep Kit*, Invitrogen) and the correct DNA structure confirmed by endonuclease restriction and DNA sequencing (Macrogen, Seoul, Korea). The constructs pSF168-87 and pSF170-87 are pIVEX2.3d derivatives carrying *lys*168-87 and *lys*170-87, respectively.

2.4.4 Production and purification of the chimerical endolysins Lys168-87 and Lys170-87

E. coli strain CG61, which overproduces phage T7 RNA polymerase upon temperate upshift (São-José *et al*., 2000) was transformed with plasmids pSF168-87 and pSF170-87 and transformants selected at 28ºC in presence of 100 μg/ml ampicillin and 40 μg/ml kanamycin. Production of soluble and active Lys168-87 and Lys170-87 by CG61 transformants was firstly confirmed by growing them over a dense lawn of autoclavated staphylococcal cells, incorporated in soft-agar LB medium, and checking for the presence of lysis halos around *E. coli* colonies (Fig. S2.2).

Selected clones of each chimera were grown at 28ºC until an optical density at 600 nm (OD_{600}) of 0.8-1.0, after which protein production was induced by changing cultures to a shaking water bath set to 42ºC. After 30min, cultures were transferred to an incubator at 16ºC and agitated for an additional period of 14h. Cells from induced cultures were pelleted by centrifugation (8,000xg, 30min, 4ºC) and resuspended in 1/50 volume of lysis

buffer (20 mM Hepes, 500 mM NaCl, 20 mM imidazole, 1% glycerol, 1 mM DTT, pH 6.5) supplemented with 1x Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche Applied Science). Cells were kept on ice and disrupted by sonication (Vibra Cell MS2T, Sonic Materials) by performing about 10 bursts of 1 min (amplitude 50, pulse 3, 30–40 W) intercalated with pauses of 1min. Insoluble material was sedimented by centrifugation (20,000xg, 30 min, 4ºC). The supernatant corresponding to the total soluble extract was filtered through 0.22μm and the chimerical enzymes purified by affinity chromatography using *HisTrap™ HP* columns (GE Healthcare) coupled to an AKTA-Prime system (GE Healthcare). The column and elution buffers had the same composition of the lysis buffer, except that the imidazole concentration in the elution buffer was 500 mM. Eluted fractions were analyzed by SDS-PAGE and Coomassie blue staining (Laemmli *et al*., 1970). Chimeras pure fractions were pooled, concentrated and changed to an imidazole-free, phosphate-based endolysin buffer (50 mM phosphate-Na, 500 mM NaCl, 25% glycerol, 1 mM DTT, pH 6.5) using *HiTrap™ Desalting* columns (GE Healthcare). Protein concentrations were determined by the Bradford method (Bio-Rad Laboratories) using bovine serum albumin as standard. The enzymes were divided in small aliquots and kept at -20ºC.

2.4.5 Evaluation of the lytic action of chimerical endolysins against bacterial pathogens

The ability of chimeras Lys168-87 and Lys170-87 to induce lysis of clinical strains from different bacterial species was evaluated by two different assays. When tested against a large number of bacterial isolates, these were individually cultured as indicated above until an OD_{600} of 0.8-1.0. Cells were recovered by centrifugation and resuspended in 1/100 volume of fresh medium. A 100μl sample of these cellular suspensions was diluted in 10ml of incorporation buffer (25 mM phosphate-Na, 250mM NaCl, pH6.5) supplemented with 0.7% agar and poured in a Petri dish. Four quantities of the purified chimeras (10, 5, 1 and 0.2 μg, in 10 μl final volume) were spotted on each bacterial lawn and after overnight incubation at 37ºC checked for the presence of lysis halos. These were evaluated and scored (- to +++) according to their relative diameter and transparency.

Bacterial cell lysis was also studied in liquid medium. Selected strains were grown until an OD_{600} of 0.3-0.4, centrifuged and cells recovered in 1/2 volumes of a phosphate buffer

(25mM phosphate-Na, 250mM NaCl, pH 6.5). One-milliliter cell suspensions were challenged with 10 μg/ml of each chimera separately or with a mixture of both enzymes, each at 5 μg/ml. The OD_{600} variation was followed over time. At the end of each assay the surviving CFU (colony forming units)/ml were determined. Negative controls were equally prepared except that endolysin buffer was added instead of endolysin.

2.5 References

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2.6 Supplementary materials

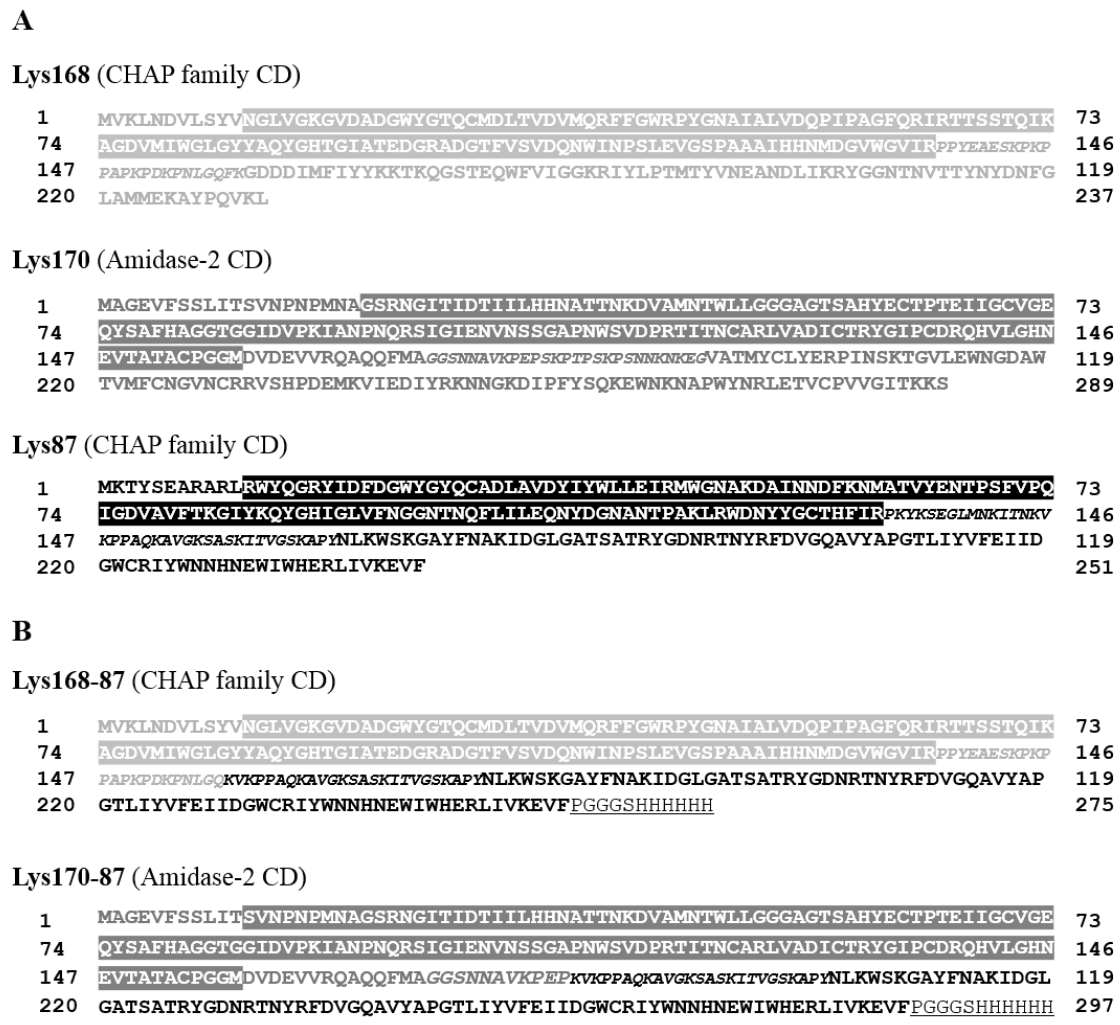
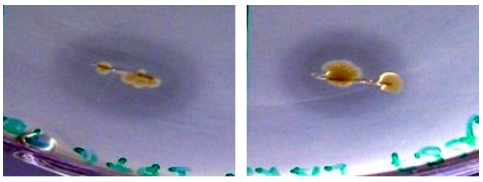


Fig. S2.1. Primary sequence of enterococcal (Lys168 and Lys170) and staphylococcal (Lys87) parental endolysins (A) and of the resulting chimeras Lys168-87 and Lys170-87 (B). N-terminal catalytic domains (CD) were defined by Pfam (http://pfam.sanger.ac.uk/) and are indicated as shaded sequences. The bioinformatics tool DLP-SVM (http://www.tuat.ac.jp/~domserv/cgi-in/DLP-SVM.cgi) and the output SVM-joint were used to define the linker domain in each endolysin (indicated by decreased-size letters and italics). Segments containing putative CW binding domains are in boldface. The C-terminal tag added by the expression vector is underlined.



Lys168-87 Lys170-87

Fig. S2.2. Production of active chimerical endolysins by *E. coli* CG61 derivatives carrying pSF168-87 and pSF170-87. CG61 derivatives were cultured in an LB soft-agar lawn containing 0.2% (w/v) of autoclavated *S. aureus* cells, 0.1% Triton-X100, 100 µg/ml ampicillin and 40µg/ml kanamycin and incubated overnight at 28ºC. Afterwards plates were incubated at 37ºC during16h. Lytic action of Lys168-87 and Lys170-87 was evidenced by development of lysis halos around CG61 colonies (Yokoi *et al*., 2005).

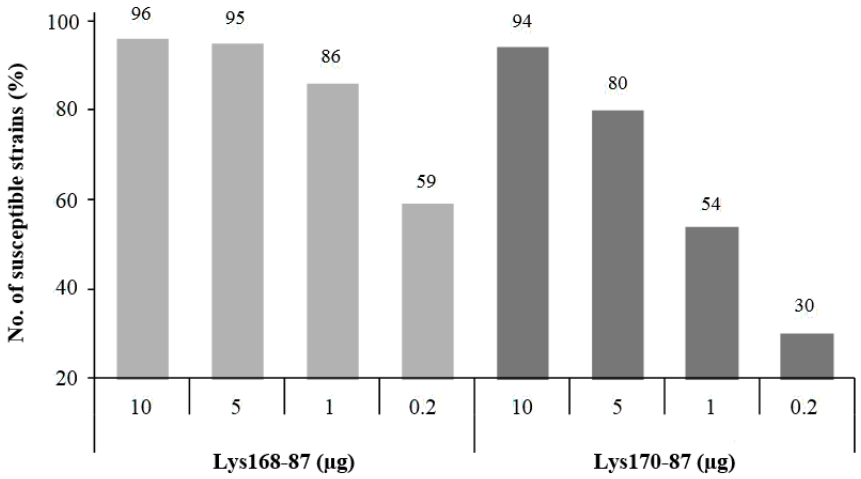


Fig. S2.3. Lytic spectrum of Lys168-87 and Lys170-87 against a panel of 100 non-typed *S. aureus* clinical isolates (Tables S2.1 and S2.2). Each isolate was grown until an OD_{600} of 0.8-1.0, cells were recovered by centrifugation and concentrated 100-fold in fresh culture medium. Samples of 100 µl of these suspensions were incorporated in a buffer (25 mM phosphate-Na, 250mM NaCl, pH6.5) supplemented with 0.7% agar and poured in a Petri dish. The indicated amounts of each enzyme were spotted on these cell lawns and plates incubated overnight at 37ºC. The percentage of isolates that presented lysis halos (Table S2.2) is plotted as a function of each chimera quantity.

Table S2.1. Non-Typed S. *aureus* Clinical Isolates

Isolate and code ID	Clinical specimens	Sample origin
		MRSA, n=42
1011/05	Wound exudate	Hospital
1018/05	Wound exudate	Hospital
1275/05	Pus from ulcer	Hospital
1463/05	Skin exudate	Hospital
1641/05	Pressure sore exudate	Hospital
1644/05	Wound exudate	Hospital
1862/05	Pressure sore exudate	Hospital
2121/05	Exudate	Hospital
124/06	Wound exudate	Hospital
623/06	Pus from phlegmon	Hospital
644/06	Foot exudate	Hospital
746/06	Ulcer exudate	Hospital
748/06	Exudate	Hospital
815/06	Exudate	Hospital
1035/06	Exudate	Hospital
1037/06	Wound exudate	Hospital
1076/06	Wound exudate	Hospital
1077/06	Exudate	Hospital
1102/06	Wound exudate	Hospital
1149/06	Exudate	Hospital
1157/06	Wound exudate	ND/NK
1201/06	Fistula exudate	Hospital
1204/06	Wound exudate	Hospital
84/07	Exudate	Hospital
161/07	Pus	Hospital
86/07	Exudate	Community
163/07	Wound exudate	Community
166/07	Purulent exudate	Hospital
400/07	Exudate	Hospital
322/07	Exudate	Hospital
162/07	Pus	Hospital
465/07	Pus	Hospital
590/07	Pus	Hospital
97/08	Pressure sore exudate	Hospital
1020/05	Synovial fluid	ND/NK
965/05	Blood	Hospital
1094/05	Blood	Hospital

Table S2.1 (continued)

2013/05	Blood	Hospital
862/06	Blood	Hospital
2030/05	Urine	Community
1007/06	Urine	Community
1211/06	Urine	Community
		MSSA, n=29
1538/05	Exudate	Hospital
1623/05	Skin exudate	Hospital
1745/05	Umbilical exudate	Hospital
1976/05	Pressure sore exudate	Hospital
399/06	Umbilical exudate	Hospital
400/06	Pus	Hospital
920/06	Exudate	Hospital
1038/06	Exudate	Hospital
1039/06	Exudate	Hospital
1156/06	Cellulitis exudate	Community
1159/06	Exudate	Hospital
1203/06	Exudate	Hospital
1209/06	Wound exudate	ND/NK
214/07	Wound exudate	Hospital
224/07	Exudate	Hospital
463/07	Exudate	Hospital
464/07	Exudate	Hospital
662/07	Exudate	Hospital
171/07	Ulcer exudate	Hospital
223/07	Exudate	Community
586/07	Exudate	Community
663/07	Exudate	Community
325/07	Exudate	Hospital
466/07	Pus	Hospital
53/08	Exudate	Hospital
55/08	Exudate	Hospital
56/08	Exudate	Hospital
129/08	Exudate	Hospital
130/08	Umbilical exudate	Hospital
		***S. aureus* (ND/NK), n= 29**
919/05	Pus	Hospital
964/05	Drain exudate	Hospital
1013/05	Catheter tip	Hospital

Table S2.1 (continued)

1133/05	Catheter tip exudate	Hospital
1152/05	Pus	Hospital
1154/05	Catheter	Hospital
1319/05	Skin lesion exudate	Hospital
1390/05	Pressure sore exudate	Hospital
1627/05	Skin exudate	Hospital
1872/05	Catheter tip	Hospital
351/06	Skin exudate	Hospital
196/07	Wound exudate	Hospital
545/07	Umbilical exudate	Hospital
547/07	Exudate	Hospital
565/07	Pharyngeal exudate	Hospital
566/07	Pharyngeal exudate	Hospital
567/07	Pharyngeal exudate	Hospital
546/07	Exudate	Community
578/07	Pressure sore exudate	Community
594/07	Exudate	Community
743/06	Exudate	Hospital
941/05	Blood	Hospital
793/06	Blood	Hospital
455/05	Urine	Community
755/05	Urine	Community
1389/05	Urine	Hospital
1649/05	Urine	Hospital
2144/05	Urine	Community
541/06	Urine	Hospital

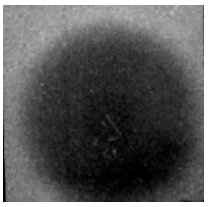
Abbreviations: ND/NK, methicillin susceptibility not determined/not known.

Table S2.2 Lytic Action of Lys168-87 and Lys170-87 Against the Group of Non-Typed *S. aureus* Clinical Isolates (n = 100)[1]

Isolates and code ID	**Lys168-87 (µg)**				**Lys170-87 (µg)**				**Illustrative**
	10	**5**	**1**	**0.2**	**10**	**5**	**1**	**0.2**	**lysis halos**
									MRSA, n=42
919/05	+++	++	+	+/-	++	+	+		
1011/05	+++	++	+	+/-	++	+	+	+/-	
1152/05	+++	++	-	-	++	+	-	-	
1319/05	++	+/-	-	-	+/-	-	-	-	
1623/05	++	+	-	-	+	-	-	-	
1627/05	++	+	-	-	+	-	-	-	

Table S2.2 (continued)

1644/05	+	+	+/-	-	+/-	+/-	-	-
1872/05	+++	++	+/-	-	-	-	-	-
1976/05	++	+	-	-	+/-	-	-	-
399/06	+++	+++	+/-	+/-	+	+/-	-	-
400/06	+/-	+/-	+/-	-	+/-	+/-	-	-
623/06	+	-	-	-	+/-	-	-	-
644/06	+++	+++	+	+/-	+	+	+/-	-
746/06	+++	++	++	+/-	+	+/-	-	-
815/06	+++	+++	+	+/-	++	+	+	-
920/06	+++	+++	++	+	+	+	+/-	+/-
1038/06	+++	++	+	+/-	+	+/-	-	-
1039/06	+++	++	++	+	++	+	+/-	-
1076/06	+++	+++	++	++	++	++	+	-
1077/06	+++	++	++	+	+	+	+/-	+/-
1149/06	+++	+++	++	+	++	+	+/-	-
1157/06	+++	+++	+++	+/-	++	++	++	-
1201/06	+++	++	++	+/-	+	+	+/-	+/-
1204/06	++	++	+/-	-	-	-	-	-
1209/06	+++	++	+	-	+	+/-	-	-
84/07	+++	+++	++	+	++	++	+	-
161/07	+++	+++	++	+	+	+	+/-	+/-
86/07	+++	++	+	+/-	+	+/-	-	-
214/07	++	++	+	+/-	+	+/-	-	-
594/07	-	-	-	-	-	-	-	-
325/07	+++	+++	++	+/-	++	++	+	-
743/06	+++	+++	++	+	+	+	+	+/-
465/07	+++	++	+	-	++	++	+	+/-
55/08	+++	+++	++	+/-	+	+	+/-	-
129/08	+++	+++	+++	++	+	+	+	+/-
1020/05	+++	++	+	+	+++	++	+	+
941/05	++	+	+/-	-	+/-	-	-	-
965/05	+++	++	+	-	+/-	-	-	-
2013/05	+++	+	+/-	-	+	-	-	-
1389/05	+++	+++	++	+	++	+	+/-	-
2144/05	+++	++	+	+/-	+	+/-	-	-
541/06	+++	+++	++	+/-	+/-	-	-	-
1390/05	+++	++	+	-	++	-	-	-
1463/05	++	+	+/-	-	++	+	+/-	-



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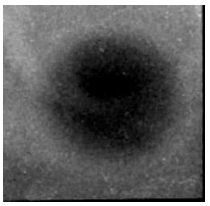

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+

MSSA, n=29

Table S2.2 (continued)

1641/05	++	+	-	-	+	-	-	-
1862/05	+++	++	+/-	-	++	+/-	-	-
124/06	+++	++	+	-	+	+	+/-	-
351/06	+++	++	+	-	+	+/-	-	-
748/06	+	+	+/-	-	+	+	-	
1035/06	+++	+++	++	+	++	++	+	
1037/06	+++	++	+	+	+++	++	+	
1102/06	+++	++	+	+/-	++	+	+/-	
1156/06	+++	+++	++	+	+	+/-	-	-
1159/06	++	++	+	+	+	+	+/-	-
1203/06	+++	+++	++	+/-	+	+/-	-	-
166/07	+	+	+/-	-	+	+	-	-
196/07	+++	+++	++	+	+	+	+	-
224/07	+	+	+/-	+/-	+/-	+/-	-	+
400/07	+++	+++	++	+	+	+	+/-	-
566/07	+++	+++	+++	+	+++	++	+	-
567/07	+++	+++	++	+	+	+	+	+/-
662/07	++	++	+	+/-	+	+/-	+/-	+/-
546/07	+++	++	+	+/-	+	+	+	-
586/07	+++	+++	++	+	+++	++	++	+/-
663/07	+++	+++	++	-	++	++	+	+
162/07	+++	+++	++	+	+	+	+	-
466/07	+++	++	+	+/-	++	+	+	+
590/07	+++	+++	++	+/-	++	++	+	-
53/08	+++	+++	++	+/-	++	++	+	+/-
56/08	++	++	+	+/-	+	+/-	-	-
97/08	+++	+++	++	-	++	+	+	-
964/05	+++	+++	+	+	+++	++	+	+/-

+/-

***S. aureus* (ND/NK), n= 29**

1013/05	+++	++	+	+/-	+	+	+/-	+/-
1018/05	+++	+++	++	+	+++	++	++	+/-
1133/05	++	+	-	-	+	+/-	-	-
1154/05	++	+	-	-	+	+/-	-	-
1275/05	+++	+	+	-	++	+	-	-
1538/05	+++	+	+/-	-	++	+	-	-
1745/05	++	-	-	-	+	-	-	-
2121/05	-	-	-	-	+/-	-	-	-
163/07	+++	++	+	+	+	+	-	-
463/07	+++	++	+	+/-	+	+	+/-	-

Table S2.2 (continued)

464/07	+++	++	+	-	+	+	+/-	-
545/07	+++	+++	++	+	++	+	+	-
547/07	+++	+++	++	+/-	++	++	+	+
565/07	++	++	+/-	-	+	+/-	-	-
171/07	+++	++	+	+/-	+	+/-	-	-
223/07	++	++	+	+/-	+/-	-	-	-
578/07	+++	+++	++	-	+	+	+	-
322/07	+	+	+	-	+/-	+/-	+/-	-
130/08	++	++	+/-	-	+	+	+	-
1094/05	+++	+++	++	++	++	++	+	+/-
793/06	++	++	+	+/-	+/-	-	-	-
862/06	-	+/-	+/-	-	-	+/-	-	-
455/05	-	-	-	-	-	-	-	-
755/05	+++	+++	++	+	++	+	+/-	-
1649/05	+++	++	+	+/-	++	+	+/-	-
2030/05	++	+	-	-	+/-	-	-	-
1007/06	++	+	+/-	-	+	+/-	-	-
1211/06	+++	+++	+++	++	+	+	+	-

-

Abbreviations: ND/NK, methicillin susceptibility not determined/not known.

[1]Four different quantities of each chimerical endolysin were spotted on a dense cell lawn of each isolate, which was prepared by incorporating cells from exponentially growing cultures in a soft-agar, phosphate-buffered medium. Lytic action was scored (- to +++) according to the relative size and transparency of lysis halos after overnight incubation at 37ºC.

Table S2.3. Lytic Action of Lys168-87 and Lys170-87 Against the Panel of Typed S. *aureus* Clinical Strains (n= 43)[1]

Strain ID	**Lys168-87 (µg)**				**Lys170-87 (µg)**			
	10	**5**	**1**	**0.2**	**10**	**5**	**1**	**0.2**
								MRSA, n=30
N315	+++	+++	++	+	+++	++	++	+
USA100	+++	++	+	+	++	+	+	+
JP1	+++	++	+	+/-	++	++	+	+/-
BM18	+++	+++	++	+	++	++	++	+
HDE288	++	++	+	+	++	+	+	+
COB3	+	+	+/-	-	+	+/-	+/-	-
USA300	++	++	+	+/-	+	+/-	+/-	-
GRE120	+++	+++	++	++	+++	+++	++	++
JH1	+++	+++	++	+	+	+	+	+
JH9	+	+/-	-	-	+/-	-	-	-

Table S2.3 (continued)

ANS46	+++	+++	++	++	+++	+++	++	+
DEN907	+++	++	+	+	++	++	+	+
HDG2	+++	++	+	+	++	++	+	+
HPV107	+	+	+	+/-	+	+	+/-	-
BK1953	++	+	+	+	+	+	+/-	-
PER184	+	+	+/-	-	+	+	+/-	-
BK793	+++	++	+	+/-	++	+	+	+/-
COL	+++	+++	++	+	++	++	+	+
MW2	++	++	+	+	++	+	+	+
USA400	++	++	+	-	+	+	+	+/-
HAR22	+	+	+/-	+/-	+	+	+	+/-
HGSA146	+++	++	+	+	+++	++	++	+
DEN2294	+++	++	+	+	++	+	+	+
DEN2946	++	+	+/-	+/-	+	+	+/-	-
USA200	+++	+++	++	+	+++	++	++	+
HAR24	++	++	+	+	++	+	+	+
HAR38	+++	+++	++	+	+++	++	++	+
WIS	++	+	+	-	+	+/-	+/-	-
GRE14	+++	++	+	+/-	+	+/-	-	-
DEN114	+++	++	+	+	++	+	+	+
								MSSA, n=13
HSA29	++	++	+	+	+	+	+/-	+/-
NCTC8325	+++	+++	++	+	+++	++	++	+
DCC403	+++	+++	++	+	++	++	+	+/-
Draftees728	++	++	+	+	++	++	+	+
IPOP33	+++	+++	++	+	+++	++	+	+
DCC192	++	++	+	+/-	++	++	+	+/-
DCC750	++	+	+	+	+++	++	+	+/-
IPOP58	+++	++	+	+/-	+	+	+	+/-
Draftees721	+++	+++	++	++	+	+/-	+/-	-
DCC1060	+++	+++	++	+	+	++	+	+
IPOP34	+++	+++	++	+/-	++	++	+	-
DCC457	+++	++	+	+/-	++	++	+	+/-
IPOP74	++	+	+	+/-	+	+	+/-	+/-

[1]Four different quantities of each chimerical endolysin were spotted on a dense cell lawn of each isolate, which was prepared by incorporating cells from exponentially growing cultures in a soft-agar, phosphate-buffered medium. Lytic action was scored (- to +++) according to the relative size and transparency of lysis halos after overnight incubation at 37ºC.

Table S2.4. Non-Typed Clinical Isolates of Gram-Positive Pathogenic Cocci other than *S. aureus*

Isolate ID	Clinical specimens
	***Staphylococcus epidermidis*, n= 10**
1736/05	Catheter tip exudate
107/08	Wound exudates
114/08	Wound exudate
158/08	Central venous catheter exudate
546/06	Urine
111/07	Urine
921/05	Catheter
222/06	Blood
223/06	Blood
224/06	Blood
	***Staphylococcus haemolyticus*, n= 9**
1318/05	Sputum
1703/05	Sputum
1930/05	Catheter tip
05/06	Blood
06/06	Urine
761/06	Blood
1065/06	Blood
198/07	Sputum
110/08	Wound exudates
	***Staphylococcus saprophyticus*, n= 7**
1847/05	Urine
1908/05	Urine
1909/05	Urine
1910/05	Urine
1911/05	Urine
424/07	Urine
123/09	Urine
	***Enterococcus faecalis*, n= 12**
926/05	Blood culture
1518/05	Pus
1551/05	Urine
1558/05	Urine
43/06	Urine
44/06	Pus

Table S2.4 (continued)

46/06	Peritoneal pus
127/06	Urine
750/06	Blood culture
751/06	Blood culture
1113/06	Blood culture
563/07	Blood culture
	***Enterococcus faecium*, n=10**
186/06	PR
187/06	PR
188/06	Blood culture
198/06	Bile
226/06	Urine
267/06	Anal swab
268/06	PR
269/06	Blood culture
389/06	Blood culture
74/07	Urine
	***Streptococcus pyogenes*, n=9**
1332/05	Wound exudates
1749/05	Vaginal exudates
2110/05	Pharyngeal exudates
422/06	Blood
790/06	Pharyngeal exudates
1070/06	Blood
54/07	Pharyngeal exudates
12/08	Pharyngeal exudates
13/08	Pharyngeal exudates

Table S2.5. Lytic Action of Lys168-87 and Lys170-87 Against Non-Typed Clinical Isolates of Gram-Positive Pathogenic Cocci other than *S. aureus*[1]

Isolates and code ID	Lys168-87 (µg)				Lys170-87 (µg)			
	10	**5**	**1**	**0.2**	**10**	**5**	**1**	**0.2**
					***Staphylococcus epidermidis*, n= 10**			
1736/05	++	+	-	-	+	+/-	-	-
107/08	+++	++	+	+/-	-	-	-	-
114/08	++	++	+	-	+/-	-	-	-
158/08	+++	+++	++	+	+	+	-	-

Table S2.5 (continued)

546/06	+++	++	+	+/-	++	++	+/-	-
111/07	+++	++	+	-	+++	+	+/-	-
921/05	++	+	+/-	-	+	+	+/-	-
222/06	+/-	+	-	-	+	-	-	-
223/06	+	+	+/-	-	+	-	-	-
224/06	++	+	+	+/-	+	+	+/-	-
				***Staphylococcus haemolyticus*, n= 9**				
1318/05	++	+	-	-	+	+/-	-	-
1703/05	++	++	+	+/-	+	+/-	-	-
1930/05	++	++	+	+	++	+	+	-
05/06	+++	++	++	+	+	+	+	+
06/06	+++	++	+	+	++	++	+	+/-
761/06	+	+	+	+/-	++	+	+	+
1065/06	+	+/-	-	-	+/-	-	-	-
198/07	+	+	+/-	-	+	+	+/-	-
110/08	++	++	+	+	++	++	+	-
				***Staphylococcus saprophyticus*, n= 7**				
1847/05	+/-	-	-	-	+	+/-	-	-
1908/05	-	-	-	-	-	-	-	-
1909/05	++	++	+	+	++	++	+	-
1910/05	++	+	+	+/-	+	+	+/-	-
1911/05	+	+	+	+/-	+	+	+/	-
424/07	+	+	-	-	+	+/-	+/-	-
123/09	+/-	+	+/-	-	+	+	+/-	-
				***Enterococcus faecalis*, n= 12**				
926/05	++	++	+	+/-	+++	+++	++	+
1518/05	+/-	+/-	-	-	++	+	+/-	+
1551/05	+	+	+/-	-	++	++	+	-
1558/05	++	+	+	+/-	+++	+++	++	+/-
43/06	+/-	-	-	-	+	+/-	+	+
44/06	++	+	+	+	+++	+++	+	-
46/06	++	++	+	+/-	+++	+++	++	+
127/06	+	+/-	+/-	-	+++	+++	++	+
750/06	+/-	-	-	-	+	-	-	-
751/06	-	-	-	-	-	-	-	-
1113/06	+	+/-	+/-	+/-	++	+	+/-	-
563/07	++	+	+	+/-	++	+	+/-	-
				***Enterococcus faecium*, n=10**				
186/06	-	-	-	-	-	-	-	-

Table S2.5 (continued)

187/06	+	+/-	-	-	+	+	-	-
188/06	+/-	-	-	-	++	+	+/-	-
198/06	+	+	+/-	-	++	+	+/-	-
226/06	+	+	-	-	+++	++	+	-
267/06	+	-	-	-	+	+/-	-	-
268/06	-	-	-	-	+	-	-	-
269/06	+	+	-	-	++	+	+	-
389/06	+/-	-	-	-	+/-	+/-	-	-
74/07	+	-	-	-	++	-	-	-
				***Streptococcus pyogenes*, n=9**				
1332/05	+	+	+	-	+	+	+	-
1749/05	+	+	+/-	-	+	+/-	-	-
2110/05	++	+	+/-	-	++	+	+/-	-
422/06	+	+	+	-	+	+	+	-
790/06	+	+	+/-	-	+/-	+/-	-	-
1070/06	+	-	-	-	+	+	+	-
54/07	+	+/-	-	-	+	-	-	-
12/08	+	+	+/-	-	++	++	+	-
13/08	++	+	-	-	+	+/-	-	-

[1]Four different quantities of each chimerical endolysin were spotted on a dense cell lawn of each isolate, which was prepared by incorporating cells from exponentially growing cultures in a soft-agar, phosphate-buffered medium. Lytic action was scored (- to +++) according to the relative size and transparency of lysis halos after overnight incubation at 37ºC.

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CHAPTER 3.

MORE THAN A HOLE: THE HOLIN LETHAL FUNCTION MAY BE REQUIRED TO FULLY SENSITIZE BACTERIA TO THE LYTIC ACTION OF CANONICAL ENDOLYSINS.

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More than a hole: the holin lethal function may be required to fully sensitize bacteria to the lytic action of canonical endolysins

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Author Disclosure Statement

The authors declare no conflict of interest.

Abstract

Double-strand DNA bacteriophages employ the holin-endolysin dyad as core components of different strategies to lyse bacterial hosts. In the so-called canonical model the holin holes play an essential role in lysis as they provide a conduit for passage of the cytoplasm-accumulated endolysin to the cell wall (CW), where it degrades the peptidoglycan. It is considered that once synthesized canonical endolysins immediately acquire their fully active conformation, having thus the capacity to efficiently cleave the peptidoglycan if contact to the CW is allowed. We show here however that holin-mediated cell death may be required to fully sensitize cells to the lytic action of canonical endolysins, a role that is obviously masked by the key function of the holin in endolysin release. We demonstrate that in certain conditions *Bacillus subtilis* cells are capable of counteracting the activity of the phage SPP1 endolysin attacking the CW either from within or from without. This capacity is lost after holin action or in presence of agents that mimic its membrane-depolarizing role. We have observed a similar relationship between lytic activity and membrane proton motive force for a staphylococcal endolysin. The possible implications of these findings in the exploitation of endolysins as enzybiotics are discussed.

3.1 Introduction

The precise regulation of host cell lysis is a key factor during phage infection. Delayed lyses can compromise the opportunity to infect new hosts whereas a premature burst results in the release of none or very few descendants (Wang *et al*., 2000). Phages with double-stranded DNA (dsDNA) genome evolved a strategy to swiftly lyse infected bacteria at the appropriate time for virion progeny release. This strategy relies on the concerted action of two fundamental phage-encoded proteins, an endolysin and a holin (Catalão *et al*., 2013; Young, 2014). Endolysins are designed to cleave the peptidoglycan, the main component of bacterial cell wall (CW), and these enzymes are instrumental for phages to achieve rapid and effective bacteriolysis (Young *et al*., 2000; São-José *et al*., 2003; Loessner *et al*., 2005). Holins are hydrophobic proteins that oligomerize in the host cell cytoplasmic membrane (CM) during the course of infection. At a genetically-programmed time holins trigger to form holes that collapse the membrane proton motive force (pmf), leading to immediate growth cessation and cell death (Gründling *et al*., 2001, Young, 2013). In the canonical lysis model, represented by the *Escherichia coli* phage λ, these holes must be large enough to allow passage of the cytoplasm-accumulated endolysin to the CW, an essential requirement for cell lysis (Young, 2002, 2014). Hole formation by canonical holins establishes therefore the onset of cell lysis by providing a pathway for endolysin access to the CW (Wang *et al*., 2000; Young *et al*., 2000).

In the last years different non-canonical lysis systems have been described where endolysins are exported to the CW compartment in a holin-independent way. For most of the studied examples, endolysin export was shown to rely on the host general secretion pathway (the Sec system), which starts to secrete the lytic enzyme to the CW very early during the viral reproductive cycle. These are the cases of phages fOg44 from *Oenococcus oeni* and P1 from *E. coli*, which produce endolysins endowed with a typical Sec-type signal peptide (SP) and a "signal-anchor-release" (SAR) domain, respectively (São-José *et al*., 2000; Xu *et al*., 2004; Young, 2014), and of the mycobacteriophage Ms6 amidase whose Sec-mediated secretion depends on the activity of a phage-encoded chaperone (Catalão *et al*., 2010). As an alternative to the Sec pathway, endolysins might be able to bind precursors of wall secondary polymers while in the cytoplasm and be co-transported during their incorporation in the CW. This seems to be the case of the endolysin of *Streptococcus pneumoniae* phage SV1, whose translocation to the CW was

proposed to be coupled to the synthesis and transport of choline-containing teichoic acids (Frias *et al*., 2013).

A key aspect of all studied phages exhibiting non-canonical release of endolysins is that they still produce holin-like proteins (Schmidt *et al*., 1996; São-José *et al*., 2004a; Frias *et al*., 2009; Catalão *et al*., 2011), a function that could be considered dispensable at first glance. However, it rapidly became clear that in these systems activity of the exported endolysins (e-endolysins) needs to be restrained to avoid premature lysis and it was found that mechanisms depending on an energized CM were responsible for counteracting the lytic action of these enzymes. In fact, in these systems holins still maintain the key role of defining the proper time for lysis thanks to their scheduled pmf-dissipating action, which directly or indirectly relieves the mechanisms restraining lytic activity of e-endolysins (Xu *et al*., 2004, 2005; Nascimento *et al*., 2008; Sun *et al*., 2009; Catalão *et al*., 2010; Frias *et al*., 2013). As previously anticipated (São-José *et al*., 2000), it was shown that the lethal character of the holin function can also result in the recruitment of host bacteria autolysins, which together with e-endolysins bring about fast and efficient lysis (Frias *et al*., 2009, 2013).

With exception of the motifs required for export, e-endolysins, canonical endolysins (c-endolysins) and autolysins can be highly related regarding primary sequence and functional domains (São-José *et al*., 2000, 2003; López *et al*., 2004; Oliveira *et al*., 2013), being the relationship between membrane pmf and lytic activity well documented for most autolysins (Rice and Bayles, 2008 and references therein, Biswas *et al*., 2012; Lamsa *et al*., 2012). Most importantly, regardless the type of lysis strategy used by dsDNA phages, one rule seems to have been conserved: endolysins do not act (or act very poorly) until infected cells are first killed by the holin function. Having all this in mind, and considering our recent observations that bacteria actively growing in rich media are able to counteract the lytic action of externally-added c-endolysins (Proença *et al*., 2015, and unpublished results), we raised the possibility that holins may have an additional role in canonical lysis, that is, that of sensitizing bacteria to the lytic action of c-endolysins. This putative function would be camouflaged by the essential role of holin holes, which provide the necessary conduit for c-endolysin release to the CW.

In this work we used the lysis system of *B. subtilis* phage SPP1, which encodes a c-endolysin, to study how conditions depressing or supporting the pmf affected lysis promoted by the endolysin reaching the CW either from the cell inside (lysis from within) or outwardly (lysis from without). The ability of a known staphylococcal c-endolysin to induced lysis from without was similarly studied. The results support a general and important function of holins in sensitizing host bacteria to the lytic action of endolysins, irrespective of the lysis mechanism.

3.2 Results

3.2.1 Actively growing *B. subtilis* cells can resist to the lytic action of secreted SPP1 endolysin

The gene encoding the SPP1 endolysin has been assigned to *orf 25* (Alonso *et al*., 1997). The deduced gp25, here called LysSPP1 harbors a putative amidase catalytic domain in its N-terminal region and a putative SH3_3 cell binding domain in its C-terminus (Nelson *et al*. 2012, Oliveira *et al*., 2013) (Fig. S3.1). LysSPP1 is predicted to be a canonical endolysin as it has no recognizable export signal that could enable its translocation to the bacterial CW (see below for results supporting this prediction). Thus, its transport to the CW must rely on the SPP1 holin function, which should be encoded by the flanking holin-like genes *24.1* and/or *26* (see below and Fig. S3.1).

The current view is that canonical endolysins accumulate in the cytoplasm in its active form, being capable of displaying lytic activity once access to the CW is provided. However, considering the hypothesis of the holin lethal function being a general requirement to turn host cells fully susceptible to the lytic action of endolysins, we wondered how *B. subtilis* growth would be affected by the intracellular production of LysSPP1 and its immediate translocation to the CW. To test this, we replaced the start codon of gene *25* by the coding sequence of the cleavable, Sec-type signal peptide (SP) of *B. subtilis* bacillopeptidase F (also known as Bpr, Sloma *et al*., 1990; Tjalsma and van Dijl, 2005). The SP of Bpr, which corresponds to the first 32 amino acid residues of the enzyme, was previously shown to promote efficient secretion of heterologous proteins (Brockmeier *et al*., 2006). The recombinant gene of the artificial e-endolysin thus generated (SP-LysSPP1) was then cloned in a replicative plasmid under the control of an

IPTG-inducible promoter and of the native translation signals of *bpr*. SP-LysSPP1 was also tagged with a hexahistidine C-terminal tail to follow its production by western blot (see methods). A similar construct producing and accumulating the native LysSPP1 in the cytoplasm was used as control.

Growth of *B. subtilis* cells carrying either the recombinant plasmids or the empty expression vector was indistinctive until mid-exponential phase, after which protein synthesis was induced by adding IPTG (time point t = 160 min in Fig. 3.1A). Remarkably, growth of the three cultures was not perturbed for at least 40 min after induction, with the cell density doubling within this period, which indicated that synthesis and Sec-mediated translocation of the potentially lethal endolysin to the CW had no obvious negative impact on actively growing *B. subtilis* cells. Cultures producing SP-LysSPP1 reached nevertheless stationary growth phase somewhat earlier than the other two (Fig. 3.1A), but no lysis was detected even after prolonged incubation (not shown).

The production of LysSPP1 and of SP-LysSPP1 precursor and mature forms 40 min after induction was confirmed by western blot analysis (Fig. 3.1B and C). The cytoplasmic LysSPP1 and the processed SP-LysSPP1 polypeptide seemed to accumulate at similar levels. However, the latter protein displayed a slightly retarded electrophoretic mobility when compared to the native endolysin. Resequencing of the constructs revealed a point mutation in the plasmid-encoded synthetic linker that connected the endolysin moiety to the hexahistidine tail in SP-LysSPP1. As result, the linker in SP-LysSPP1 had the amino acid sequence PGGDS, whereas in LysSPP1 it had the expected PGGGS sequence. Although not impairing enzyme activity (see below), this substitution might be responsible for the slightly different migrations of the processed SP-LysSPP1 and LysSPP1.

The extracytoplasmic localization and lytic character of the processed form of SP-LysSPP1 was readily evidenced when half of the 40-min-induced cultures was treated with gramicidin D ("G" in Fig. 3.1A). Gramicidin D is an ionophore that dissipates the electrical and pH gradients of the pmf. It inserts in the CM and forms small-sized channels that are only permeable to monovalent cations such as K^+, Na^+ and H^+ (Harold, 1972; Jolliffe *et al*., 1981; Kelkar and Chattopadhyay, 2007). In this, as well as in the assays described below, the gramicidin concentration and growth conditions allowed the drug to

exert its pmf-dissipating action without causing significant autolysis during the course of the experiments. As expected, addition of gramicidin to the three cultures led to immediate growth interruption. However, rapid and extensive cell lysis could only be observed in the cultures producing SP-LysSPP1, which was a strong indication that the endolysin was secreted and accumulated extracellularly. Actively growing cells synthesizing either LysSPP1 or SP-LysSSP1 did not release detectable amounts of endolysin polypeptides to culture supernatant (Fig. S3.2). This suggested tight association of the SP-LysSPP1 mature form with the CW after translocation and SP removal (see below). The fact that addition of the ionophore to cultures synthesizing LysSPP1 resulted in none or very poor lysis supported the canonical character of the SPP1 endolysin. Interestingly, stopping the aeration of cultures that accumulated SP-LysSPP1 was sufficient to evoke lysis, although not as efficiently as with gramicidin (Fig. S3.3).

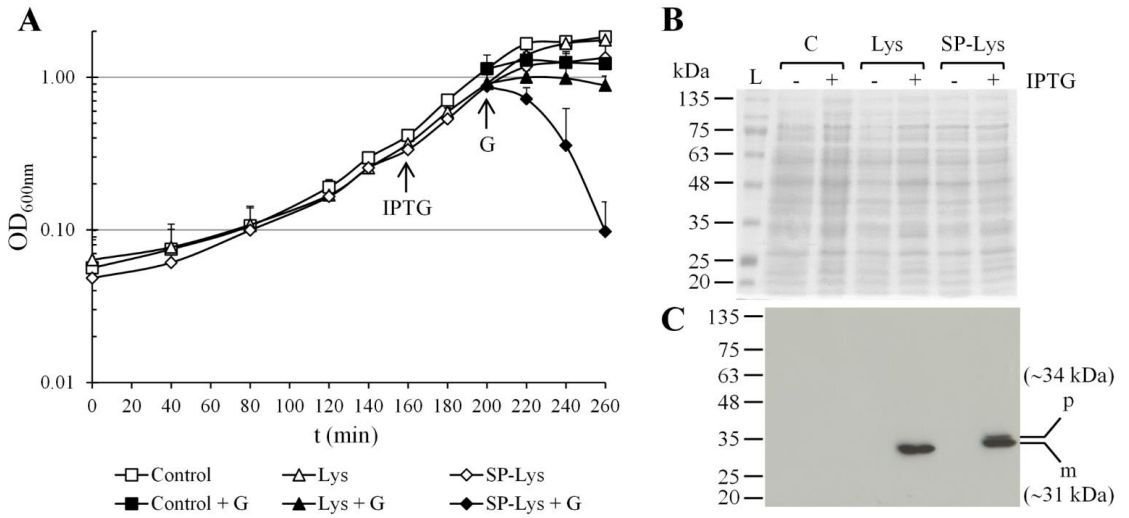

Fig. 3.1. Production of the phage SPP1 endolysin in *B. subtilis* and effect on cell growth. (**A)**. Exponentially growing cells harboring the empty expression vector (Control) or derivatives producing LysSPP1 (Lys) or SP-LysSPP1 (SP-Lys) were IPTG-induced at the indicated time and growth followed until late exponential phase. At this point, gramicidin (G) was added to half of the cultures (filled symbols) and cultures optical density at 600 nm (OD_{600nm}) followed for 60 min. Each curve is the average of at least three independent assays. (**B)**. Coomassie blue-stained, SDS-PAGE gel analyzing equal amounts of total protein extracts produced from cultures described in panel A at time point t = 200 min (before gramicidin addition), in absence (-) or presence of IPTG (+). The molecular weight of bands composing the protein ladder (L) is indicated on the left side. (**C)**. Detection by western blot analysis of the SPP1 endolysin polypeptides in the protein extracts described in panel B, using anti-His_6 specific antibodies. Note the production of precursor (p) and mature (m) forms in the cultures producing SP-LysSPP1 (SP-Lys).

In conclusion, the results showed that as long as *B. subtilis* cells are maintained in an energized state they can cope with secretion to the CW of the potentially lethal SPP1 endolysin. With pmf dissipation, cells become susceptible to the lytic action of the enzyme.

3.2.2 LySPP1-mediated lysis from without is enhanced in conditions leading to CM depolarization

We asked next if the *B. subtilis* cell energy state could also affect susceptibility to LysSPP1 lytic action when the endolysin reaches the CW from the outside (from without). LysSPP1 was overproduced in *E. coli* with its C-terminus fused to a His_6 tag and subsequently purified by affinity chromatography (see methods and Fig. S3.4A). *B. subtilis* cells at mid-exponential growth phase were two-fold concentrated in fresh LB medium and two sample sets were prepared in spectrophotometer cells. One set was supplemented with gramicidin while the other received the ionophore solvent. After 10 min equilibration, cells from both sets were challenged with different concentrations of purified LysSPP1 (ranging from 0.5 to 10 µg/ml) and lysis monitored under static conditions by following the optical density (OD_{600nm}) of cell suspensions.

In absence of the ionophore the lowest LysSPP1 concentrations (0.5 and 1 µg/ml) failed to elicit any measurable cell lysis during the time course of the assays (Fig. 3.2A), despite efficient endolysin binding to cells in these conditions (Fig. S3.5). Cell lysis could be detected for the remaining endolysin concentrations (2.5, 5 and 10 µg/ml), with the rate and extent of OD_{600nm} decrease correlating with the enzyme concentration. Interestingly, in analogous experiments with cultures maintained under permanent shaking cells revealed to be much more resistant to lysis (see below). When cells were treated with gramicidin before LysSPP1 addition, the three highest endolysin concentrations produced fastest and more extensive lysis and the enzyme dose effect became less obvious (Fig. 3.2B). Most importantly, lysis could also be detected for the two lowest LysSPP1 concentrations.

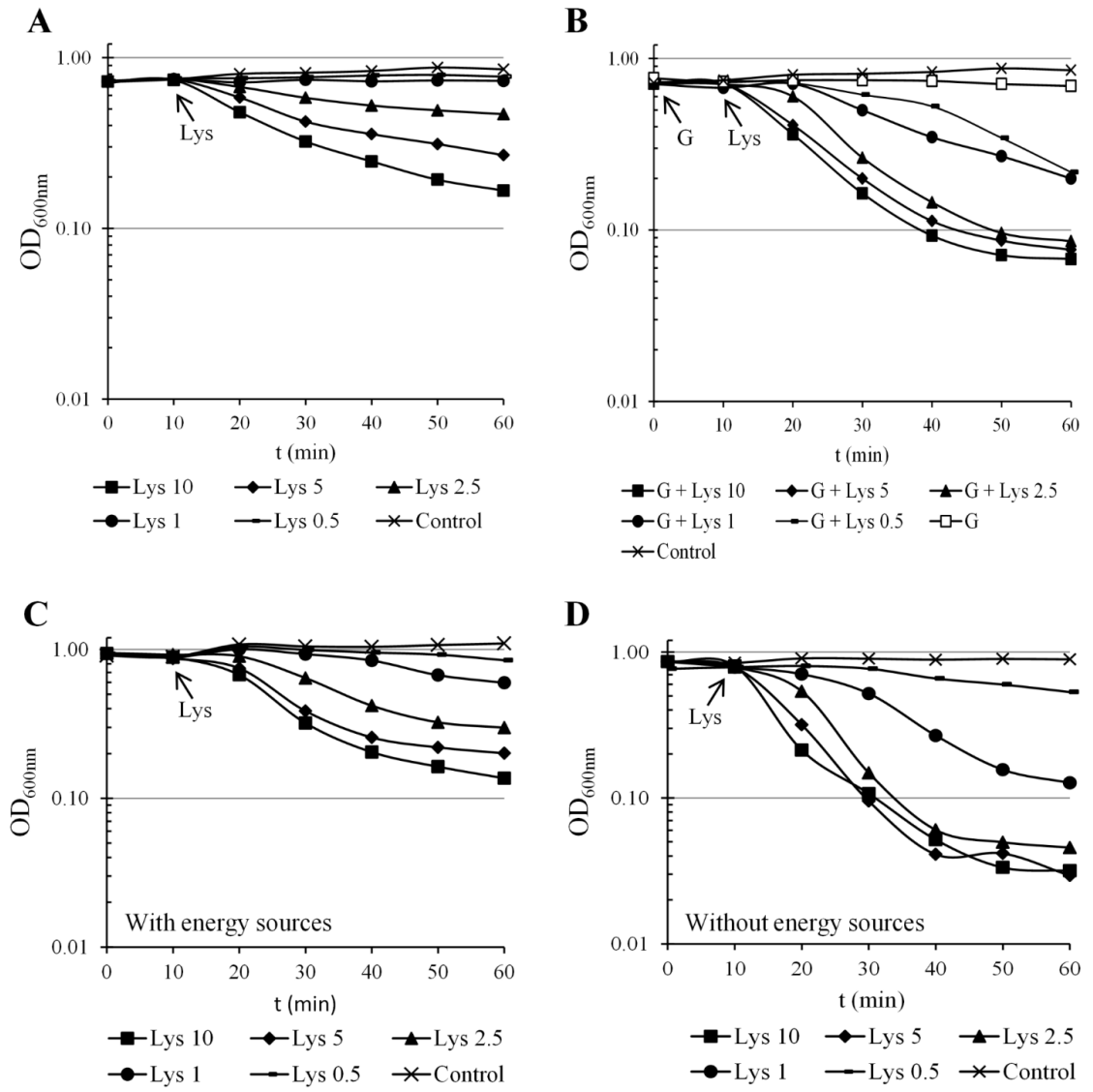

Fig. 3.2. *B. subtilis* lysis from without under pmf-supporting and pmf-dissipating conditions. (**A)** and (**B)**. Cells from exponentially-growing cultures were recovered in fresh LB medium in absence (A) or presence (B) of gramicidin ("G" in panel B). After 10 min equilibration, cells were challenged with LysSPP1 (Lys) at the indicated concentrations (µg/ml) and lysis monitored under static conditions. Controls with cells only ("Control" curves) or with cells plus gramicidin ("G" curve) were similarly prepared. Each curve is the average of at least three independent assays. For clarity, error bars were omitted. (**C)** and (**D)**. Exponentially-growing cells were collected in minimal medium with (C) or without (D) energy sources. After 10 min equilibration, cells were challenged with LysSPP1 and lysis monitored as described above. Controls with cells only ("Control" curves) were similarly prepared. Each curve is the average of at least three independent assays. Error bars were omitted for clarity.

Bacterial sensitization to lysis upon gramicidin treatment could result from a specific effect of this ionophore or be a general response to any stress causing dissipation of the membrane potential. To test if other conditions compromising pmf resulted also in lysis enhancement, cell suspensions were prepared as above but using minimal medium with or without energy sources, instead of LB medium (see methods). After 10 min equilibration, LysSPP1 was added as described above and lysis monitored over time. In presence of energy sources, lysis profiles for the different endolysin concentrations were similar to those observed in complex medium in absence of gramicidin (Fig. 3.2A and C). On the other hand, in the condition leading to collapse of the pmf (no energy sources), lysis was akin to that registered in presence of gramicidin (Fig. 3.2B and D). We should also note that nisin, a lantibiotic that induces pore formation in the CM with concomitant membrane depolarization (Hasper *et al*., 2004; Tol *et al*., 2015) behaved essentially as gramicidin in promoting lysis from without by LysSPP1 (data not shown).

Overall, the results indicated that conditions leading to dissipation of the membrane pmf render cells more susceptible to lysis from without.

3.2.3 LySPP1 concentration requirements in lysis from within and from without

Results from the previous sections suggested that *B. subtilis* lysis by LysSPP1 in absence of the holin function, or of agents mimicking its pmf-dissipating action, could require significantly higher amounts of the enzyme. In order to have an estimation of the endolysin quantities operating in the context of SPP1 infection we constructed a mutant phage producing LysSPP1 fused to a hexahistidine tag (SPP1*g25His*, see methods). Phage SPP1*g25His* plated with normal plaque morphology and lysed *B. subtilis* liquid cultures within the expected time, indicating that the phage growth parameters were not significantly affected (not shown).

B. subtilis cultures infected with the mutant phage at mid-exponential growth phase (OD_{600nm} = 0.3-0.4) started to lyse at an OD_{600nm} of about 0.8 (Fig. 3.3A), which was the cell density set for the lysis assays described previously (Figs 3.1A and 3.2). As showed above, lysis of *B. subtilis* cells producing SP-LysSPP1 could be elicited by adding gramicidin ("SP-Lys + G" curve in Fig. 3.1A). The superimposition of the lysis curve

obtained in presence of the ionophore with that resulting from SPP1*g25His* infection suggested comparable lysis kinetics in both conditions (Fig. 3.3A).

Western blot analysis indicated that immediately before lysis onset, LysSPP1 content of infected cells was slightly lower than the amount detected in cells producing SP-LysSPP1 from the recombinant plasmid (Fig. 3.3B and C). To estimate the level of endolysin production in these two scenarios of lysis from within, the intensities of the corresponding LysSPP1 bands and that of bands resulting from known quantities of purified endolysin (Fig. 3.3B and C) were analyzed by densitometry. After correcting for the cell concentration factor inherent to the production of protein extracts (50-fold), we estimated about 0.3 and 0.4 µg of LysSPP1 produced per milliliter of culture lysed from within after SPP1*g25His* infection and after production of SP-LysSPP1, respectively. To lyse from without exponentially growing cultures with identical cell densities, under the same aerated conditions, we needed to add 30 µg/ml of LysSPP1 to the cultures (Fig. 3.3A). This concentration could be reduced 60-fold in presence of gramicidin (Fig. 3.3A), in agreement with the results reported above.

Although factors like enzyme local concentration probably facilitate lysis from within in the two scenarios studied here (SPP1*g25His* infection and SP-LysSPP1 in presence of gramicidin), the results strongly suggest that cells with dissipated pmf require much lower endolysin amounts for lysis, both from within or from without. Therefore, it is most likely that in the context of phage infection the holin action results in a stimulation of endolysin activity, in addition to its essential role in releasing the lytic enzyme to the CW

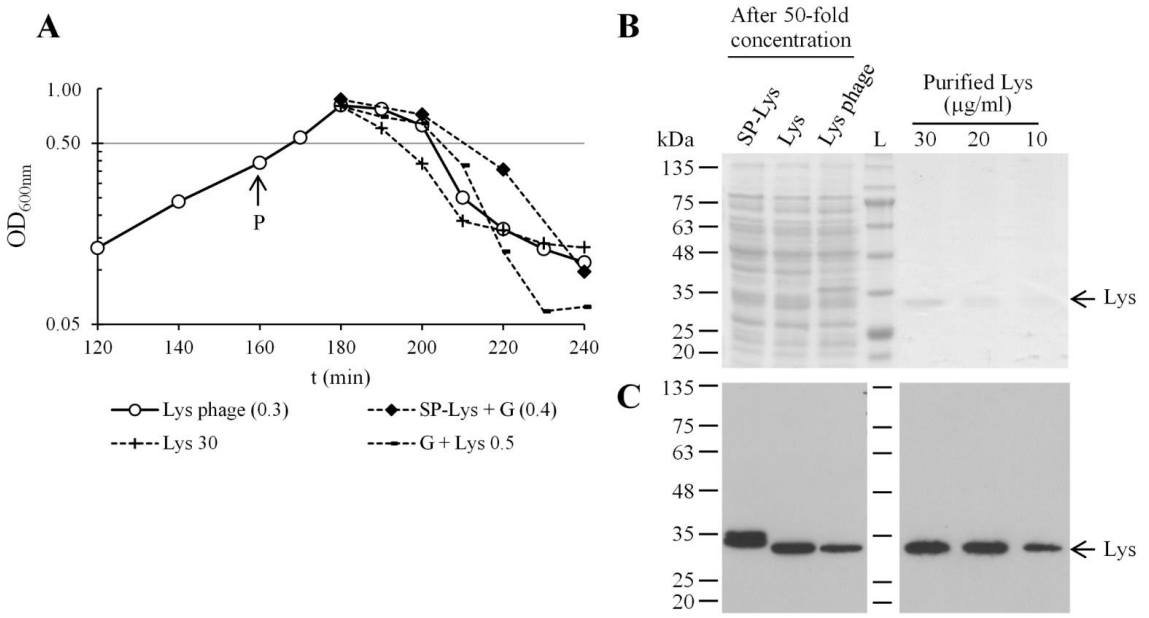

Fig. 3.3. LysSPP1 quantities in lysis from within and from without. (A). Representative lysis curve of a *B. subtilis* culture infected with phage SPP1*g25His* ("Lys phage" curve). The culture was infected at the indicated time point (P) and immediately before lysis onset (t = 180 min) a sample was collected for endolysin detection by western blot (panels B and C). For lysis profile comparison, 3 curves (dashed lines) were overlaid on that of infected cells using as reference the time point just before lysis start: the curve "SP-Lys + G" from Fig. 3.1A and two curves of lysis from without obtained after treating cells with gramicidin plus 0.5 µg/ml LysSPP1 (G + Lys 0.5) or with 30 µg/ml LysSPP1 only (Lys 30). Note that here lysis from without occurred under aerated conditions. The indicated endolysin concentrations for the "Lys phage" and "SP-Lys + G" curves (0.3 and 0.4 µg/ml, respectively) are rough estimations based on densitometry analysis of endolysin bands of panel C. (**B)**. Coomassie blue-stained, SDS-PAGE gel of total protein extracts prepared from the SPP1*g25His*-infected culture (Lys phage) and from cultures producing SP-LysSPP1 (SP-Lys) or LysSPP1 (Lys) (see Fig. 3.1). The 3 extracts were produced from culture samples collected immediately before lysis onset (OD_{600nm} ~0.8) and after 50-fold cell concentration. Ten microliters of these extracts and of preparations of purified LysSPP1 at the indicated concentrations (right side of the protein ladder, L) were run in parallel. The molecular weight of bands composing the protein ladder is indicated on the left side. (**C)**. Detection of LysSPP1 polypeptides in the protein samples described in panel B using anti-His_6 specific antibodies. Note that the endolysin bands in lanes "SP-lys", "Lys" and "Lys phage" are detected in extracts prepared from 50-fold-concentrated cell suspensions.

3.2.4 Expression of SPP1 holin-like genes in *B. subtilis* increases cell susceptibility to LysSPP1

Lysis enhancement by gramicidin (see above) was interpreted as the ionophore being able to mimic the pmf-dissipating action of the SPP1 holin function, resulting somehow in increased susceptibility of the depolarized *B. subtilis* cells to LysSPP1. To have a more direct link between holin action and sensitization to endolysin-mediated lysis, we wanted to study how expression of the SPP1 holin function in *B. subtilis* affected its capacity to counteract LysSPP1 attack. In the annotated SPP1 genome sequence the holin function is predicted to be encoded by *orf 26*, which follows immediately downstream of the endolysin gene (Alonso *et al*., 1997 and Fig. S3.1). However, as noticed previously (Catalão *et al*., 2013) the gene product of *orf 24.1*, which lays immediately upstream of the endolysin-holin pair also has holin-like features. In fact, gp24.1 and gp26 share sequence homology and overall predicted membrane topology with the holin-like proteins XlhA and XhlB, respectively, which together with the amidase XlyA constitute the lysis cassette of the *B. subtilis* defective prophage PBSX (Fig. S3.1). Krogh *et al*. (1998) observed that expression in *B. subtilis* of either XhlA or XhlB in pairwise combinations with the XlyA did not result in the expected host cell lysis phenotype. This was only observed when the three genes were co-expressed, leading the authors to propose that in PBSX the holin "functional unit" allowing endolysin release is most probably a membrane complex made of XhlA and XhlB.

A hallmark of the holin function is its ability to convert into lethal holes after reaching a critical concentration in the membrane (Young, 2013). We cloned in a replicative plasmid the SPP1 holin-like genes *24.1* and *26* as a transcriptional fusion, under the control of a xylose-inducible promoter (see methods). *B. subtilis* cells carrying the transcriptional fusion presented delayed growth when compared to cells harboring the empty expression vector and 10 min xylose-induction was sufficient to cause almost 2-log reduction of cell viability (~3 x 10^7 and ~6 x 10^5 cfu/ml immediately before and after induction, respectively). We are currently studying the contribution of each gene to the observed lethal phenotype, but the available results suggest that the SPP1 holin function likely requires the concerted action of the two holin-like proteins, as observed for PBSX.

In agreement with our hypothesis, cells killed by the SPP1 holin(s) were rapidly and extensively lysed from without with as low as 0.5 µg/ml of purified LysSPP1 (Fig. 3.4A),

a protein concentration that caused no measurable lysis of healthy bacteria in analogous conditions (Fig. 3.2A). Yet, the same amount of endolysin slightly lysed control cultures that were not induced for holin expression (Fig. 3.4A). This suggested leaky expression of *24.1-26* in absence of xylose, something that fitted also the already mentioned delayed growth of the cultures in absence of the inducer. Somewhat unexpectedly, 10 min induction of *24.1-26* expression was sufficient to support significant cell lysis in absence of the endolysin after transferring cultures to static conditions (Fig. 3.4A). In contrast to previous assays with gramicidin (Fig. 3.2B), here the holin action seemed to rapidly activate the *B. subtilis* autolytic machinery (Jolliffe *et al*., 1981; Kemper *et al*., 1993; Lamsa *et al*., 2012). This was confirmed when we performed the same experiment in a *B. subtilis* background deleted of the major autolysin LytC (Kuroda and Sekiguchi, 1991; Blackman *et al*., 1998). In these new conditions the sole expression of *24.1-26* did not induce significant lysis (Fig. 3.4B), although it still resulted in cell death (confirmed by the decrease of viable cell counts, data not shown). As expected, these killed cells were susceptible to lysis with 0.5 µg/ml of LysSPP1 in contrast to control cell suspensions (non-induced cultures treated with the same endolysin concentration) (Fig. 3.4B). We have tried also to study the effect of *24.1-26* expression during lysis from within, that is, in cells producing either SP-LysSPP1 or LysSPP1. However, we were unable to stably maintain the constructs expressing both the endolysin and holin genes within the same *B. subtilis* cell, probably due leaky transcription in absence of the inducers.

In conclusion, the results confirmed that *B. subtilis* cells killed by the SPP1 holin function lose the capacity to restrain LysSPP1 lysis from without. In addition, holin-induced CM depolarization seems to recruit host cell autolytic enzymes that contribute to lysis. A similar observation was previously reported for the holin of the pneumococcal phage SV1 (Frias *et al*., 2009, 2013).

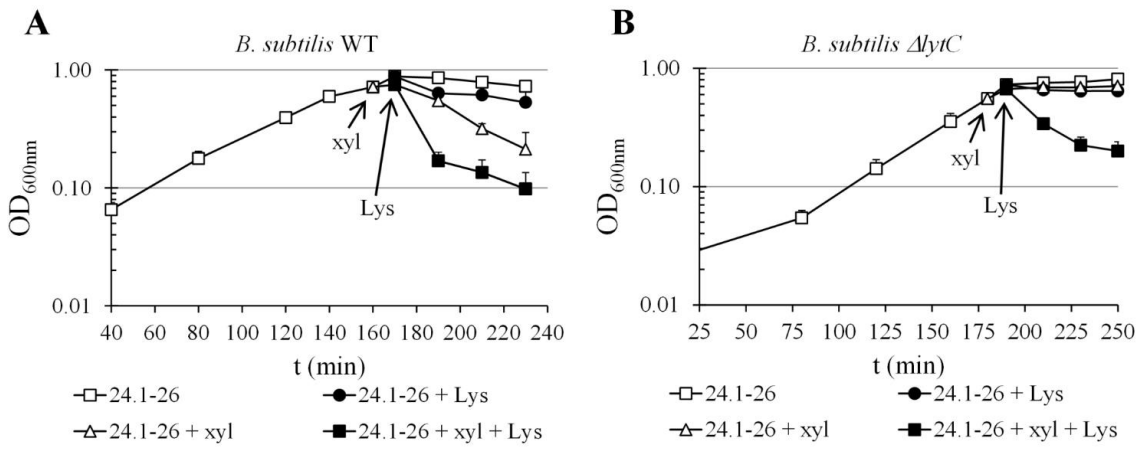

Fig. 3.4. **Effect of holin activity on *B. subtilis* susceptibility to LysSPP1 endolysin. (A)**. Cells carrying a transcriptional fusion of the SPP1 holin-like genes *24.1* and *26* (24.1-26) under control of a xylose-inducible promoter were grown until exponential growth phase and the culture split in two halves. One half was induced for 10 min with xylose (xyl) after which 0.5 µg/ml of LysSPP1 (Lys) were added (filled symbols). The optical density of cell suspensions was then followed for 60 min in static conditions. Controls consisted of non-induced cultures added of the same concentration of LysSPP1 and of induced cultures added of the equivalent volume of endolysin buffer. Each curve is the average of at least three independent assays. (**B).** Same assay as in panel A except that carried out in a *B. subtilis ΔlytC* genetic background.

3.2.5 Lytic action of the staphylococcal ϕ11 endolysin is drastically incremented in gramicidin-treated cells

Finally, we wanted to see if the increased sensitivity of *B. subtilis* cells to endolysin-mediated lysis promoted by gramicidin could be extended to other Gram-positive bacteria. Multidrug-resistant *S. aureus* is a widely known pathogen whose infections are becoming increasingly difficult to treat, and endolysins from staphylococcal phages have been proposed as potential therapeutic alternatives to fight this bacterium (Schmelcher *et al*., 2015). One such endolysin is that of phage ϕ11, a two-catalytic domain enzyme displaying endopeptidase and amidase activities (Navarre *et al*., 1999, Sass and Bierbaum, 2007).

As for LysSPP1, we purified the ϕ11 endolysin (here called Lys11) as a hexahistidine-tagged protein (Fig. S3.4B) and tested its lytic action against *S. aureus*. Cells from mid-exponential growth phase were collected in half volume of fresh culture medium and

challenged with 20 μg/ml of Lys11, either in absence or presence of gramicidin. Lysis was followed under static or aerated conditions. Standing cell suspensions suffered some lysis (less than 50% decrease of the initial OD_{600nm}), which could be greatly increased if cells were first treated with gramicidin (Fig. 3.5A). This followed therefore the same lysis pattern observed for LysSPP1 (Fig. 3.2A and B). Remarkably however, if *S. aureus* cultures were kept under permanent agitation in absence of gramicidin they were completely resistant to Lys11 attack and quickly resumed growth (Fig. 3.5B). Under aerated conditions Lys11-mediated lysis was thefore dependent on pre-treatment of target cells with the ionophore (Fig. 3.5B).

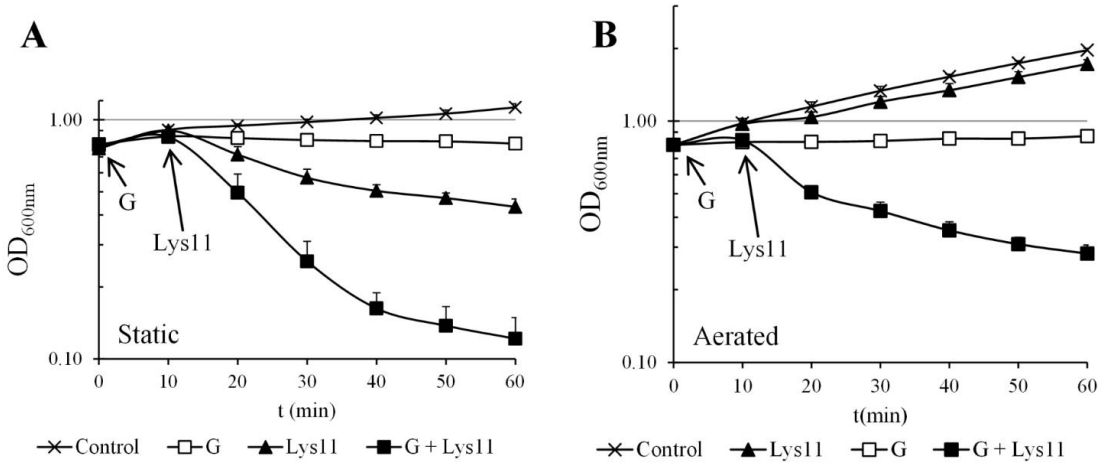

Fig. 3.5. Lysis of *S. aureus* by the ϕ11 endolysin under pmf-supporting and pmf-dissipating conditions. (A). Cells from exponentially-growing cultures were recovered in fresh TSB medium in absence or presence of gramicidin (G). After 10 min equilibration, cells were treated with 20 μg/ml of the endolysin (Lys11, filled symbols) and lysis monitored under static conditions. Controls with cells only ("Control" curve) or with cells plus gramicidin ("G" curve) were similarly prepared. Each curve is the average of at least three independent assays. (**B)** Same as in panel A except that performed under aerated conditions.

We concluded therefore that the lytic action of different endolysins can be facilitated when the pmf of target cells is first dissipated. In addition, the level of "resistance" to lysis of actively growing, fully energized cells may vary according to the bacterium/endolysin pair.

3.3 Discussion

Phage holins are commonly described has having two major roles in canonical lysis: i) to define the end of phage infection by killing host cells through membrane hole formation, and ii) allow escape of the cytosol-accumulated endolysins through these holes for CW degradation. These two interconnected functions define the timing of cell lysis. In this work we present several evidences supporting that holin activity can have an additional important role in canonical lysis, which is to sensitize cells to the lytic action of endolysins. Naturally, this role is concealed by the essential function of holins in releasing c-endolysins to the CW. We showed that *B. subtilis* cells could grow almost normally when secreting to the CW the phage SPP1 c-endolysin, which was equipped with a host cell Sec-type SP for that purpose. The exported, processed form of the enzyme accumulated to similar or even higher levels than those observed for the native c-endolysin during phage infection, indicating that the secreted enzyme remained in an "inactive" state after translocation and SP removal. Addition of a pmf-dissipating agent immediately elicited endolysin activity and cell lysis, in striking similarity to the general lysis features of known e-endolysins. These results seem to challenge the accepted view that c-endolysins are designed to instantly degrade the peptidoglycan no matter the pathway followed to reach the CW. In other words, our results indicate that activity of c-endolysins can be restrained by other means besides their physical separation from the CW, imposed by the cytoplasmic membrane, as postulated by the canonical lysis model.

We did not rule out the possibility that secretion guided the endolysin to particular regions on the CW where its activity could be tightly controlled. This would provide an alternative explanation to our results, assuming that this preferential localization and/or control cannot occur when c-endolysins are released to the CW through the holin holes. However, we showed that dissipating the membrane pmf by different means increased also cell susceptibility to the c-endolysins added from without. This suggests that mechanisms restraining endolysin activity are generally operating in the CW as long as the cell pmf is maintained. Since the simple membrane de-energization by nutrient exhaustion or by permeation to small ions was sufficient to increase c-endolysin activity, we concluded that it is the holin pmf-dissipating action that boosts enzyme activity, and not its ability to promote the release of eventual cytoplasmic factors activating endolysins. It was previously shown for at least two other predicted c-endolysins that treatment of target

cells with the pore-forming lantibiotic nisin resulted in a drastic enhancement of lytic activity (García *et al*., 2010, Proença *et al*., 2015).

Triggering of lytic activity upon holin-inflicted CM damage is well established for the e-endolysins with SAR export signal and for the putative amidase of *S. pneumoniaee* phage SV1, an e-endolysin which is believed to be translocated to the CW along with choline-containing teichoic acids (Frias *et al*., 2013). In the former case, e-endolysins are exported by the host cell Sec system but the SAR sequence retains them in the periplasm as inactive forms tethered to the CM. Dissipation of the pmf after holin trigger releases the N-terminal SAR domain from the CM, which is followed by conformational and/or intramolecular chemical changes that generate the active, soluble forms of the endolysins in the periplasm (Xu *et al*., 2004, 2005, Sun *et al*., 2009; Kuty *et al*., 2010).

For the SV1 amidase and the e-endolysins known to be secreted by the Sec system in Gram-positive and mycobacterial systems (São-José *et al*., 2000; Catalão *et al*., 2010) it is still unknown how the observed or assumed holin action leads to endolysin activation. What seems clear is that the mechanisms restraining e-endolysin activity are abolished upon dissipation of the host cell membrane pmf. For this reason, it has been speculated (São-José *et al*., 2000; Nascimento *et al*., 2008; Catalão *et al*., 2010; Frias *et al*., 2013) that e-endolysin activity on the CW could be controlled by the same processes that regulate bacterial endogenous peptidoglycan hydrolases, some of which (autolysins) are well-known for lysing bacteria in response to conditions that collapse the pmf (Jolliffe *et al*., 1981; Kemper *et al*., 1983; Moreillon *et al*., 1990; Bayles, 2000; Martínez-Cuesta *et al*., 2000; Lamsa *et al*., 2012). The exact mechanisms modulating autolysin activity, and which probably regulate the abovementioned e-endolysins and the c-endolysins studied here, are poorly understood. Several studies though have implicated the level of protonation (pH) of the CW compartment, which is directly linked to the pmf, and its possible effects on the conformation, distribution and/or chemical composition of secondary CW polymers in such regulation (Calamita *et al*., 2001; Neuhaus and Baddiley, 2003; Rice and Bayles 2008; Biswas *et al*., 2012).

As already mentioned, the results suggest that under pmf-supporting conditions bacteria may counteract the activity of some c-endolysins, likely by the same mechanisms that regulate autolysin and e-endolysin activities. This is somewhat expected if we consider

the evolutionary relationship between phage endolysins and host cell peptidoglycan hydrolases (São-José *et al*., 2000, 2003; López *et al*., 2004; Oliveira *et al*., 2013). The rule "holins (or other pmf-dissipating agents) first kill and only then cell lysis occurs" is common to c- and e-endolysin-mediated lysis and also to different autolysis phenomena. It appears thus that a great number of peptidoglycan hydrolases have evolved to be fully effective only against energy-depleted bacteria. Theoretically, lysis of phage-infected cells can result in the release of endolysins to the surrounding medium, which might be harmful to neighbour bacteria lacking an external membrane. If these bacteria are potential hosts for new rounds of infection, it can be advantageous to phages that the pmf-dependent mechanisms controlling lytic activity can also operate against c-endolysins reaching the CW from without.

Phage endolysins have been extensively explored as potential antimicrobial agents to fight antibiotic resistant Gram-positive pathogens (for reviews see Nelson *et al*., 2012; Schmelcher *et al*., 2012; Pastagia *et al*., 2013). The underlying idea is that these enzymes should be able to efficiently lyse viable bacteria when added exogenously in the form of purified proteins. In apparent conflict with the ideas conveyed here, a great number of *in vitro* studies (fewer *in vivo*), including some emanating from our group (Fernandes *et al*., 2012; Proença *et al*., 2012) seem to support the lytic efficacy of endolysins added externally to Gram-positive bacteria. However, a large number of these studies have been performed in conditions that do not support bacterial growth, i.e., before endolysin challenge cells are typically washed and suspended in nutrient-depleted, buffered solutions which are unable to sustain normal pmf. Still, although our study shows that the lytic efficacy of c-endolysins can be dramatically improved upon pmf dissipation, it also suggests that the capacity and limits of energized cells to counteract endolysin attack may vary depending on growth conditions and on the particular bacterium/endolysin pair. This should account for those examples where c-endolysins proved effective *in vitro* against bacteria in growth-promoting conditions and *in vivo* (for reviews see Nelson *et al*., 2012; Pastagia *et al*., 2013). The take home message is that when exploring endolysins as enzybiotics one should be aware that their killing efficacy may be significantly affected by the energetic state of target cells.

In summary, our findings indicate that "activation" of endolysin lytic activity as result of the holin action may be a general feature in bacterial lysis mediated by dsDNA phages,

independently of the pathway used to deliver the lytic enzyme to the CW. As suggested recently (Proença *et al*., 2015), this should be taken in consideration as it may be necessary to choose or engineer endolysins that can bypass this holin sensitization requirement, and thus achieve maximal killing efficacy.

3.4 Materials and methods

3.4.1 Bacterial strains, phages and growth conditions

Bacterial strains and phages used in this study are indicated in Table S3.1. *B. subtilis* and *E. coli* were routinely grown in LB medium (Sambrook and Russell, 2001) with orbital shaking (220 rpm), whereas *S. aureus* was propagated in tryptic soy broth (TSB), also with aeration. Culture media components were purchased from Biokar Diagnostics, France. *B. subtilis* was pre-cultured overnight (ON) at 30ºC, whereas ON growth of *E. coli* cloning strains and *S. aureus* was at 37ºC. New cultures were initiated by diluting pre-cultures 100-fold in fresh media. For details on the growth conditions of the *E. coli* expression strain CG61 (São-José *et al*., 2000) and its derivatives see below. When selecting for DNA constructs, LB medium was supplemented with appropriate antibiotics such as ampicillin (100 µg/ml), kanamycin (40 µg/ml) or chloramphenicol (20 µg/ml) for *E. coli* strains, and kanamycin (20 µg/ml), neomycin (7.5 µg/ml), chloramphenicol (5 µg/ml) and erythromycin (0.5 µg/ml) for *B. subtilis* strains. Bacterial growth was monitored by taking OD_{600nm} measurements at regular intervals. SPP1 phages were propagated in *B. subtilis* strain YB886 as described previously (Jakutytè *et al*., 2011). When indicated, YB886 cultures at an OD_{600nm} of 0.4 (~5.0 x 10^7 cfu/ml) were infected with phages at an input multiplicity of 10.

3.4.2 General DNA techniques

Plasmids and primers used in this work are listed in Tables S3.1 and S3.2, respectively. Standard recombinant DNA techniques were applied to construct all plasmids used in this study (Sambrook and Russell, 2001). Extraction of *B. subtilis* and phage DNA was as reported previously (São-José *et al*., 2004b; Vinga *et al*., 2012). High-fidelity polymerase chain reaction (PCR) was carried out with Kod Hot Start Master Mix (Novagen, USA), whereas routine PCR was performed with DNzyTaq green 2x master mix (NZYTech,

Portugal). Different commercial kits (Roche Applied Science, Germany and NZYTech) were used for purification of plasmid DNA and DNA fragments (PCR products or DNA restriction fragments). When necessary, DNA fragments were extracted from agarose gels with the Wizard SV gel and PCR Clean-Up System (Promega, USA). FastDigest restriction enzymes (Fermentas, Thermo Ficher Scientific, USA) were used to cleave DNA according to the manufacturer instructions. T4 DNA ligase and the 1 kb Plus DNA Ladder were from Invitrogen (Thermo Fisher Scientific, USA). Development of competence and transformation of *E. coli* and *B. subtilis* strains was as described by Chung *et al*. (1989) and Yasbin *et al*. (1975), respectively. Constructs were confirmed by DNA sequencing (GATC Biotech, Germany).

3.4.3 General protein techniques

For production of *B. subtilis* total protein extracts 2 or 4 ml-samples were collected from cultures at OD_{600nm} = 0.8 and cells recovered in 1/50 volumes of lysis buffer (50 mM Tris.Cl pH 7.5, 300 mM NaCl, 10 mM $MgCl_2$, 100 µg/ml lysozyme and 10 µg/ml DNase I) supplemented with a protease inhibitor cocktail (1x Complete Mini EDTA-free Protease Inhibitor Cocktail, Roche Applied Science). After 15 min at 37ºC, cell lysates were put on ice and supplemented with 0.1% NP-40. Protein quantification was carried out with the Bradford reagent (Bio-Rad Laboratories, USA) with bovine serum albumin (Bio-Rad Laboratories) as standard. Total protein extracts or endolysin purification fractions (see below) were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting as described elsewhere (Sambrook and Russell, 2001). The NZYColour Protein Marker II (NZYTech) or the BenchMark Pre-Stained Protein Ladder (Invitrogen, Thermo Fisher Scientific) were used as protein molecular weight markers. His_6-tagged endolysin proteins were immunodetected with 0.3-0.4 µg/ml of anti-His6 antibodies (Roche Applied Science), with antigen/antibody complexes being revealed either with the BM Chemioluminescence Western Blotting Kit (Roche Applied Science) or with the Luminata Forte Western HRP Substrate (Merck Millipore, USA).

3.4.4 Cloning and expression of endolysin genes in *E. coli*

Genes *25* and *53*, which encode the endolysins LysSPP1 and Lys11 of phages SPP1 and ϕ11, respectively (Acc. No. X97918 and NC_004615), were PCR amplified from the corresponding phage DNA using the primer pairs Gp25-NcoI/Gp25-XmaI (for gene *25*) and Gp53-NcoI/Gp53-XmaI (for gene *53*). The amplified DNA fragments were double-digested with *Nco*I/*Xma*I and ligated to the equally digested expression vector pIVEX2.3d (Roche Applied Science). This vector allows expression of cloned genes under the control of the phage T7 ϕ10 promoter and the production of the corresponding proteins C-terminally fused to a hexahistidine tail. The resulting recombinant plasmids pIV::25His and pIV::53His were obtained in *E. coli* strain XL1-Blue MRF' and then transferred to the *E. coli* expression strain CG61, which produces phage T7 RNA polymerase upon temperate up-shift (São-José *et al*., 2000). CG61 cells carrying pIV::25His (CG/pIV::25His) or pIV::53His (CG/pIV::53His) were selected and maintained at 28ºC until induction of protein synthesis (see next).

3.4.5 Production and purification of His_6-tagged endolysins

E. coli strains CG/pIV::25His and CG/pIV::53His were used for heterologous production of endolysins LysSPP1 and Lys11, respectively. Both strains were pre-cultured ON at 28ºC and then the protocols followed to produce and purify endolysins differed. CG/pIV::25His cultures were 100-fold diluted in fresh LB medium and further grown at 28ºC until OD_{600nm} of 0.8-1.0. After a 10-min period at room temperature in static conditions, protein production was induced by incubating cultures for 30 min at 42ºC in a shaking water bath. Induced cultures were then transferred to an orbital incubator and growth continued at 37ºC for 2.5 h. Cells were pelleted by centrifugation (8,000 *g*, 30 min, 4ºC) and resuspended in 1/50 volumes of lysis buffer (50 mM Hepes-Na, pH 7.0, 500 mM NaCl, 0.1% Triton, 1% glycerol and 50 mM imidazole) supplemented with the protease inhibitor cocktail. Cells were disrupted by sonication and the endolysin purified by metal chelate affinity chromatography (Fig. S3.4A) as described previously (Fernandes *et al*., 2012). LysSPP1 pure fractions were pooled, concentrated and finally exchanged to an imidazole-free buffer (same composition of lysis buffer but without imidazole) using a HiPrep 26/10 Desalting column (GE Healthcare, UK).

Production and purification conditions were optimized to improve solubility of Lys11 endolysin. After ON growth, CG/pIV::53His cultures were 100-fold diluted in LB medium prepared in 0.1 M phosphate buffer pH 7.2 and supplemented with 0.5 M D-sorbitol. Cells were grown at 28ºC until OD_{600nm} of 0.5, after which cultures were supplemented with 80 mM KCl and protein synthesis induced as explained above. Induced cultures were transferred to a shaking water bath set to 16ºC and incubated for 12-14 h. Cells were recovered by centrifugation and resuspended in 1/50 volume of lysis buffer (50 mM Hepes-Na, pH 6.8, 500 mM NaCl, 10 mM $MgCl_2$, 0.1% Triton, 1 mM TCEP, 10 % glycerol and 50 mM imidazole) supplemented with the protease inhibitor cocktail. Cells were disrupted in a French press and Lys11 purified as described above (Fig. S3.4B). Lys11 was kept in an imidazole-free buffer (same composition of lysis buffer but without imidazole).

Purified preparations of both endolysins lacked significant amounts of large protein aggregates as revealed by analytical size-exclusion chromatography in a column Superdex 200 5/150 GL column (GE Healthcare). Purified endolysins were kept at – 80ºC as small aliquots of ~20 mg/ml (LysSPP1) and ~2 mg/ml (Lys11). Before use, aliquots were centrifuged (15,000 *g*, 30 min, 4ºC) to eliminate eventual aggregates formed during storage.

3.4.6 Cloning and expression of SPP1 lysis genes in *B. subtilis*

LysSPP1 and SP-LysSPP1. The SPP1 endolysin gene fused to the hexahistidine coding sequence was PCR-amplified from plasmid pIV::25His with the primer pair lysSPP1Fw/pIV23d-His-Eco and cloned between the *Spe*I and *Eco*RI sites of the *E. coli* vector pBluescript II KS(+) (pKS, Stratagene, USA). The *25His* DNA fragment was then recovered from the resulting plasmid pKS::25His through *Xba*I/*Sal*I cleavage and inserted in the equally digested *E. coli/B. subtilis* shuttle vector pDG148 (Stragier *et al*., 1988), originating plasmid pDG::25His. As the restriction site *Xba*I in pDG148 is blocked by Dam methylation, the vector was prepared from a *dam E. coli* strain (SCS110, Stratagene). Genes cloned in pDG148 are expressed from the IPTG-inducible promoter P_{spac}. To construct the gene encoding the artificial e-endolysin SP-LysSPP1, two independent PCR products were first generated. One corresponded to the SP coding sequence and native ribosome binding site (RBS) of the serine protease Bpr (Acc. No.

NC_000964), which was amplified from the *B. subtilis* genome with primers SPbprFw and SPbprRv. The other matched the *25His* sequence, starting from the second codon, and was amplified with the primer pair SPbprLYSfw/pIV23d-His-Eco using pIV::25His as template. The DNA segments SP_{bpr} and *25His* carried 3' and 5' complementary ends, respectively, which allowed their fusion by overlap-extension (OE)-PCR in a reaction employing primers SPbprFw and pIV23d-His-Eco. The OE-PCR product was cloned between the *Spe*I and *Eco*RI sites of pKS, originating pKS::SP-25His. Finally, the *SP-25His* DNA segment was released from the latter plasmid with *Xba*I/*Sal*I and inserted in pDG148, yielding pDG::SP-25His. Plasmids pDG::25His and pDG::SP-25His were first obtained in *E. coli* strain XL1-Blue MRF' and then transferred to *B. subtilis* strain YB886, originating YB/pDG::25His and YB/pDG::SP-25His, respectively. An YB886 derivative carrying the empty pDG148 (YB/pDG) was used as control in the indicated experiments. Gene expression in YB886 strains carrying the pDG148 derivatives was induced with 1 mM IPTG when cultures reached mid-exponential growth phase (OD_{600nm} = 0.3-0.4). After reaching an OD_{600nm} of 0.8-1.0, a fraction of the induced cultures was treated with 5 µg/ml of gramicidin from *Bacillus brevis* (Prod. No. G5002, Sigma-Aldrich, USA) and cell lysis monitored for 60 min. Stock solutions of gramicidin (2 or 10 mg/ml) were prepared in absolute ethanol.

SPP1 holin-like genes. To express the SPP1 holin-like genes *24.1* and *26* (Fig. S3.1) we constructed first the expression vector pNPxyl, which is a derivative of the *E. coli*/*B. subtilis* shuttle vector pNW33N (Mee and Welker, unpublished, Acc. No. AY237122) carrying the XylR repressor and the xylose-inducible promoter P_{xylA} from *B. megaterium* (Kim *et al*., 1996). The *xylR*-P_{xylA} cassette together with the upstream terminator t_0 from phage λ were amplified from plasmid pAX01 (Härtl *et al.*, 2001) with primers Pxyl-Mfe and Pxyl-Kpn. The resulting fragment was inserted between the *Eco*RI and *Kpn*I sites of pNW33N, resulting in pNPxyl. Next, the primer pairs gp24.1SPP1fw/gp24.1-holSPP1rev2 and gp24.1-holSPP1fw/holSPP1rv were used to amplify genes *24.1* and *26*, respectively, from the SPP1 DNA. The two PCR products carried complementary ends allowing the transcriptional fusion of *24.1* and *26* by OE-PCR with primers gp24.1SPP1fw and holSPP1rv. The fusion *24.1-26* maintained the native translation signals for both genes. The DNA fragment *24.1-26* was digested with *Kpn*I and *Cla*I and ligated to the equally digested pMutin-cMyc (Kaltwasser *et al*., 2002), resulting in the translation fusion of the 3' end of gene *26* to the coding sequence of the c-Myc epitope.

The DNA segment *24.1-26cMyc* was PCR-amplified from the ligation reaction using the primer pair gp24.1SPP1fw/HAcMycrv, the PCR product was cleaved with *Kpn*I and *Sph*I and finally inserted into the similarly digested pNPxyl. The resulting plasmid pNP::24.1-26cMyc was directly obtained in *B. subtilis* YB886 in presence of 0.1% of glucose, yielding strain YB/pNP::24.1-26cMyc. Plasmid pNP::24.1-26cMyc was also used to transform a YB886 derivative deficient for the major autolysin LytC (YBΔLytC), giving rise to strain YBΔLytC/pNP::24.1-26cMyc. Strain YBΔLytC was obtained by transforming the recipient strain YB886 with DNA from the donor *ΔlytC* strain L16648 (Margot *et al*., 1999), and selecting for neomycin resistance. YB886 strains harboring pNPxyl derivatives were grown until OD_{600nm} of 0.6-0.7 and expression of SPP1 holin-like genes induced in half of the cultures by adding 0.5% xylose. Cultures were maintained in growth conditions (37ºC with shaking) for 10 min, after which they were distributed to 1-ml spectrophotometer cells. The cell suspensions were then challenged with 0.5 μg/ml of purified LysSPP1 or the equivalent volume of endolysin protein buffer. OD_{600nm} was monitored during 60 min after endolysin addition.

3.4.7 Construction of an SPP1 phage producing a His_6-tagged endolysin

The SPP1 endolysin gene *25* together with the 3' hexahistidine-coding tail was PCR-amplified from pIV::25His with primers lysSPP1FwSac and pIV23d-His-Sal, and the resulting PCR product cloned between the *Sac*I and *Sal*I sites of the *E. coli*/*B. subtilis* shuttle vector pGKV259 (Kok *et al*., 1984). The resulting plasmid pGK::25His was isolated in *E. coli* strain TG1. A second PCR product encompassing gene *26* (including its translation signals) and the first 127 bp of the downstream gene *27* was amplified from the SPP1 DNA with primers HolSPP1fwSal and gp27rvNsi. This DNA fragment was digested with *Sal*I/*Nsi*I and inserted between sites *Sal*I and *Pst*I of the previously constructed pGK::25His. The resulting plasmid pGK::25His-26-27' was isolated in *B. subtilis* YB886, giving rise to strain YB/pGK::25His-26-27'. This strain served as host to propagate the wild type (WT) phage SPP1 in solid media and thus promote recombination events between the phage DNA and homologous regions carried in pGK::25His-26-27'. At each round of infection, phages from a confluent lysis plate were recovered with 3ml TBT buffer (100 mM Tris.Cl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$) an the relative proportion of recombinant phages qualitatively assessed by PCR using 1 μl of serial dilutions of the lysates and the primer pairs gp24.1SPP1fw/lysSPP1rv (detects WT and

recombinant phages) and gp24.1SPP1fw/pIV2.3d-His-Sal (detects recombinant phages only). After 4 rounds of infection the proportion of the mutant phage in the lysate appeared close to 10%. This lysate was then plated in the plasmid-free WT strain YB886 and 19 pools of 5 isolated phage plaques each (total of 95 plaques) were similarly analyzed by PCR. One of the positive pools was again diluted and plated in strain YB886 and 10 isolated phage plaques screened individually, revealing 5 clearly positive for the His-tagged endolysin gene. Finally, one plaque of the recombinant phage (SPP1*g25His*) was used to produce a medium scale lysate, with purity of the recombinant phage being controlled by PCR at each amplification step.

3.4.8 Assays of lysis from without

Target cells from exponentially-growing cultures (OD_{600nm} = 0.4) were recovered in half volume of fresh medium (LB for *B. subtilis* and TSB for *S. aureus*) and for each cell suspension two sample sets were prepared in 1-ml spectrophotometer cuvettes. One set was supplemented with gramicidin (5 µg/ml for *B. subtilis* and 30 µg/ml for *S. aureus*) while the other received the equivalent volume of the ionophore solvent. After 10 min equilibration at 37ºC, cells from both sets were challenged with the indicated endolysin concentrations (or the equivalent volume of endolysin buffer) and lysis monitored in static conditions by taking OD_{600nm} measurements at defined time points after endolysin addition. Analogous assays were performed where the LB medium was substituted by complete GM1 minimal medium (Yasbin *et al*., 1975) or GM1 lacking the energy sources (0.5% glucose, 0.1% yeast extract and 0.02% acid-hydrolyzed casein). Lytic activity was also tested against cells actively growing under aerated conditions when cultures reached an OD_{600nm} of 0.8, both in absence or presence of gramicidin.

3.4.9 Bioinformatics tools

Protein homologies were revealed by BLASTP analysis (Altschul *et al*., 1997) using NCBI (National Center for Biotechnology Information, USA) non-redundant protein sequence database. Protein conserved domains were identified with tools CDD (Marchler-Bauer *et al*., 2015) and HHpred (Söding *et al*., 2005). Putative transmembrane helices were predicted with TMHMM (Krogh *et al*., 2001). Signal quantification of western blot bands was carried out with ImageJ (Schneider *et al*., 2012).

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3.6 Supplementary materials

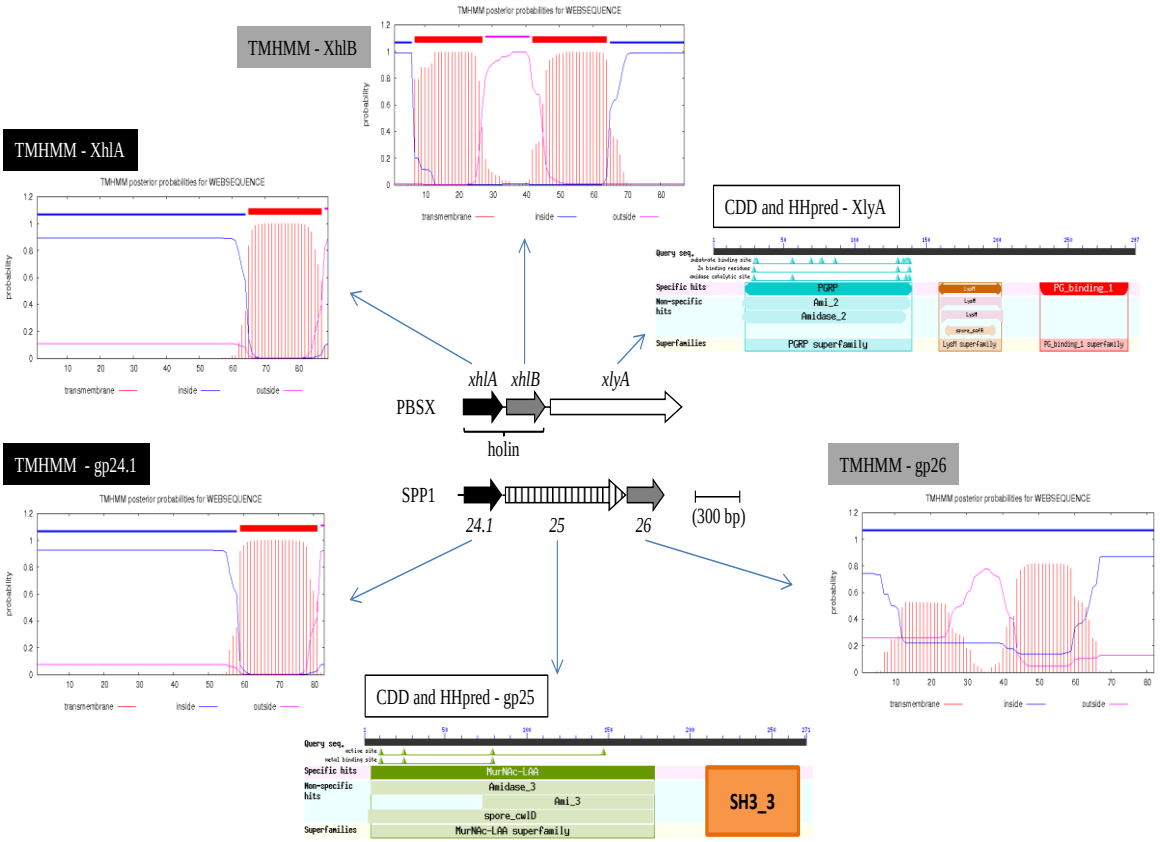

Fig. S3.1. Lysis genes of *B. subtilis* phages PBSX and SPP1. Representation of the genome segments of phages PBSX (Acc. No. NC_000964) and SPP1 (Acc. N0. X97918) encompassing the endolysin (white or stripped arrows) and holin-like (black or gray arrows) genes. Genes encoding homologous proteins have the same filling pattern. Conserved domains were identified with tools CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and HHpred (http://toolkit.tuebingen.mpg.de/hhpred). Putative transmembrane helices in gene products were predicted with TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). XhlA and gp24.1 are members of the Pfam family XhlA (pfam10779), which includes cell-surface associated haemolysins. XhlB and gp26 are members of the Pfam family Phage_holin (pfam04688), which includes several holin-like proteins from Siphoviridae bacteriophages. Endolysin XlyA exhibits 3 conserved domains, a catalytic Amidase_2 domain (pfam01510) and the cell wall binding motifs LysM (pfam01476) and PG_Binding_1 (pfam01471). The CDD tool identified a single conserved domain, the catalytic Amidase_3 (pfam01520) in gp25 (LysSPP1). The SH3_3 domain (PF08239) in the gp25 C-terminus is only revealed by HHpred analysis (probability = 95.9%, E-Value = 0.15).

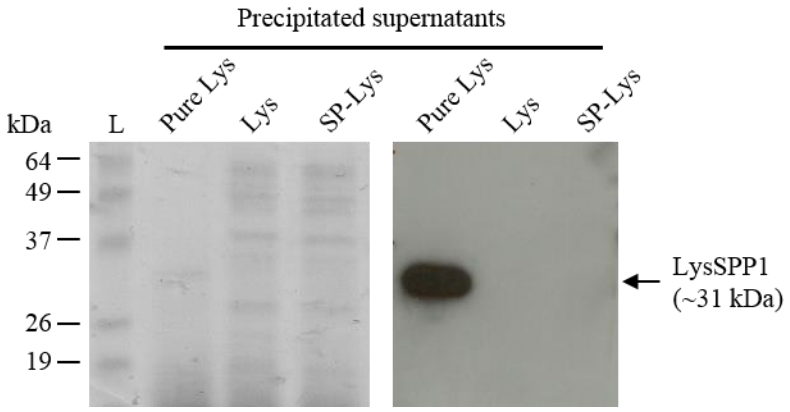

Fig. S3.2. Endolysin detection in the supernatants of *B. subtilis* cultures producing LysSPP1 or SP-LysSPP1. Cultures synthesizing LysSPP1 (Lys) or SP-LysSPP1 (SP-Lys) were collected at a time point equivalent to t = 200 min of Fig. 3.1A and the corresponding cell-free supernatants concentrated 100-fold by TCA-DOC precipitation. As control of the procedure, purified LysSPP1 (Pure Lys) set to a concentration of 0.2 µg/ml in LB medium was similarly precipitated. Ten microliters of the solubilized precipitate (in 50 mM Tris.Cl pH 8.8, 7 M urea and 2 M thiourea) were analyzed by SDS-PAGE/Coomassie blue staining (left panel) and by western blot using anti-His_6 antibodies (right panel, overexposed film).

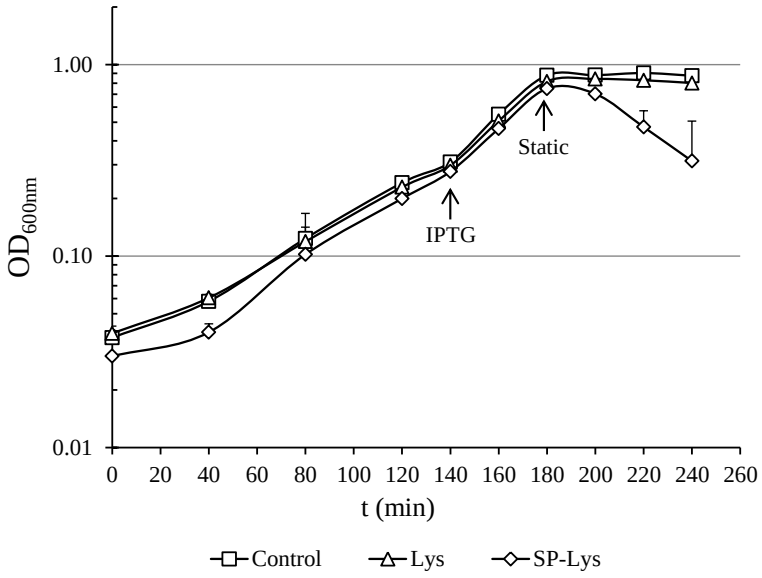

Fig. S3.3. Effect of aeration on *B. subtilis* resistance to secreted SP-LysSPP1. Exponentially growing cells harboring the empty expression vector (Control) or derivatives producing LysSPP1 (Lys) or SP-LysSPP1 (SP-Lys) were IPTG-induced at the indicated time and growth allowed for 40 min under orbital shaking. After this point, samples of the cultures were transferred to spectrophotometer cells and lysis of cell suspensions monitored under static conditions. Each curve is the average of at least three independent experiments.

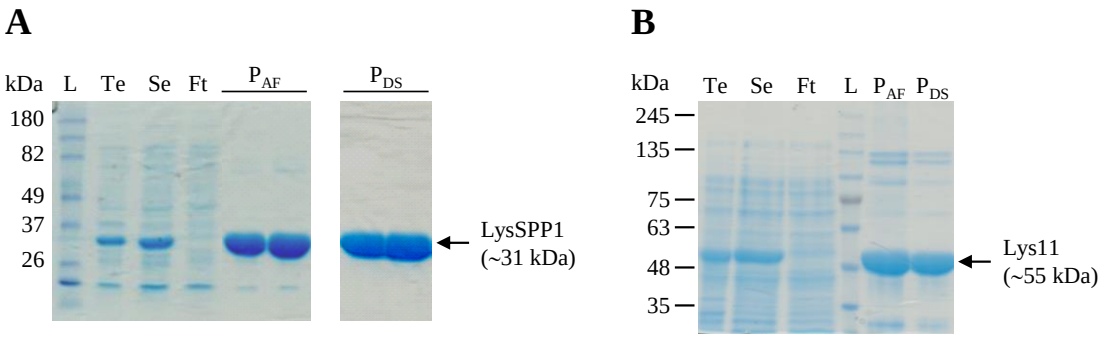

Fig. S3.4. SDS-PAGE analysis of different steps of endolysins LysSPP1 (A) and Lys11 (B) purification by affinity chromatography (AF). L, Protein ladder; Te, total protein extract; Se, soluble protein extract loaded in the AF column; Ft, flowthrough of the AF column; P_{AF}, AF peak fractions; P_{DS}, desalting peak fractions. The molecular weight of bands composing the protein ladders is indicated on the left side.

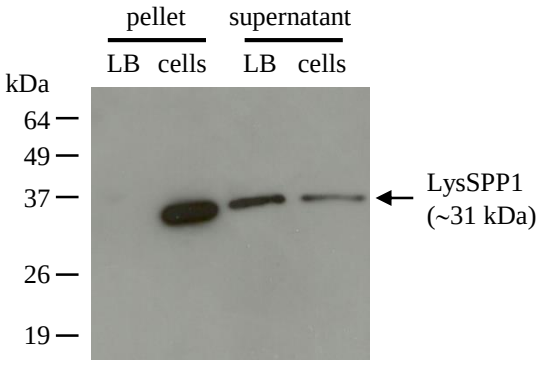

Fig. S3.5. LysSPP1 binding to *B. subtilis* cells. Cells from a 4-ml sample of an exponentially growing culture of *B. subtilis* strain YB886 (OD_{600nm} = 0.4) were recovered in 2 ml of fresh LB medium ("cells" lanes) and transferred to a BSA-coated microtube (Proença *et al*., 2015). For control purposes, a BSA-coated microtube was filled with the same volume of fresh LB only ("LB" lanes). Both tubes were then supplemented with 0.5 µg/ml of pure LysSPP1 and incubated for 30 min at room temperature, with occasional tapping of the tubes. After this period, tubes were centrifuged for cell sedimentation, washed with fresh LB and resuspended in 50 µl of lysis buffer ("pellet" fractions, with cells lysed as described in text). The 2-ml supernatants were filtered through a 0.25 µm membrane and concentrated to 50 µl by passing through a 10 kDa-cutoff Centricon (Millipore) ("supernatant" fractions). Ten microliters of pellet and supernatant fractions were analyzed by western blot using anti-His_6 antibodies. Note that nearly all added LysSPP1 specifically co-sedimented with cells, indicating that the endolysin efficiently binds to *B. subtilis* in the conditions of Fig. 3.2A.

Table S3.1. Bacterial Strains, Phages and Plasmids Used in This Study

Bacteria, Bacteriophages and Plasmids		
	Genotype and/or relevant features	**Reference[1] /Source**
		***E. coli* strains**
XLIBlue-MRF'	*Δ*(*mcrA*)*183 Δ*(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI*^{q} *lacZΔM15* Tn*10* (Tet^{r})]; cloning host	Stratagene
TG1	*supE thi-1 Δ*(*lac-proAB*) *Δ*(*mcrB-hsdSM*)*5,* ($r_K^-m_K^-$) [F' *traD36 proAB+ lacIq lacZΔM15*]; cloning host	Stratagene
SCS110	*rpsL* (Str^{r}) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ*(lac-proAB)* [F′ *traD36 proAB lacI*^{q} *lacZ*Δ*M15*]; for preparation of plasmid free of Dam or Dcm methylation	Stratagene
CG61	BL21 derivative harboring pGP1-2 (kan^{r}); allows T7 RNA polymerase production upon temperature upshift	9
CG/pIV::25His	CG61 derivative harboring pIV::25His	This work
CG/pIV::53His	CG61 derivative harboring pIV::53His	This work
***B. subtilis* strains**		
L16648	*lytABC*::*neo lytD*::*tet lytE*::*cam lytF*::*spc*; multiple autolysin-deficient mutant	6/BGSC[2]
YB886	*B. subtilis* 168 derivative cured of prophages PBSX and SPβ; wild-type strain; SPP1 indicator strain	11
YB/pDG::25His	YB886 derivative harboring pD::25His	This work
YB/pDG::SP-25His	YB886 derivative harboring pDG::SP-25His	This work
YB/pDG	YB886 derivative harboring pDG148	This work
YB/pNP::24.1-26cMyc	YB886 derivative harboring pNP::24.1-26cMyc	This work
YBΔLytC	YB886 derivative *lytABC*::*neo*; deficient for the major autolysin LytC	This work
YBΔLytC/pNP::24.1-26cMyc	YBΔLytC derivative harboring pNP::24.1-26cMyc	This work
YB/pGK::25His-26-27'	YB886 derivative harboring pGK::25His-26-27'	This work
		***S. aureus* strains**
RN4220	prophage cured, restriction-deficient mutant of strain 8325-4	1, 5
		Bacteriophages
SPP1	*B. subtilis* strictly lytic phage	8
SPP1g25His	SPP1 derivative carrying the 3' end of endolysin gene *25* fused to a His_6 tag sequence	This study

Table S3.1 (continued)

		Plasmids
pIVEX2.3d	*E. coli* expression vector; genes expressed from T7 ϕ10 promoter and proteins synthesized with a C-terminal His_6 tag; Amp^r	Roche Applied Science
pBluescript II KS(+) (pKS)	*E. coli* cloning vector; Amp^r	Stratagene
pDG148	*E. coli*/*B. subtilis* shuttle vector; genes expressed from the IPTG-inducible promoter P_{spac}; Amp^r Kan^r	10/BGSC[2]
pNW33N	*E. coli/B. subtilis* shuttle vector; Cm^r	Mee and Welker, unpublished/ BGSC[2]
pAX01	*E. coli* replicative/*B. subtilis* integrative vector; source of the *B. megaterium* expression cassette t_0-*xylR*-P_{xylA}; Amp^r Ery^r	2/BGSC[2]
pMutin-cMyc	*E. coli* replicative/*B. subtilis* integrative vector; source of cMyc epitope; Amp^r Ery^r	3/BGSC[2]
pGKV259	*E. coli/B. subtilis* shuttle vector; Ery^r Cm^r	4
pIV::25His	pIVEX2.3d derivative carrying SPP1 endolysin gene *25*	This work
pIV::53His	pIVEX2.3d derivative carrying ϕ11 endolysin gene *53*	This work
pKS::25His	pKS derivative carrying the *25His* coding sequence from pIV::25His	This work
pKS::SP-25His	pKS derivative carrying SP_{bpr} fused to *25His* from pIV::25His	This work
pDG::25His	pDG148 derivative carrying *25His* from pKS::25His	This work
pDG::SP-25His	pDG148 derivative carrying *SP-25His* from pKS::SP-25His	This work
pNPxyl	Expression vector; pNW33N derivative carrying the expression cassette t_0-*xylR*-P_{xylA}	This work
pNP::24.1-26cMyc	pNPxyl derivative carrying a transcriptional fusion of the SPP1 holin-like genes *24.1* and *26*	This work
pGK::25His	pGKV259 derivative carrying *25His* from pIV::25His	This work
pGK::25His-26-27'	pGKV259 derivative carrying *25His* from pIV::25His transcriptionally fused to gene *26* and the first 127 bp of gene *27*.	This work

[1]The references corresponding to the numbers indicated in the Table can be found in section 3.6.1; [2]Bacillus Genetic Stock Center, Ohio State University, USA.

Table S3.2. List of Primers Used in This Work

Name	Sequence (5' → 3')[1]
Gp25-NcoI	GAAAGA**CCATGG**GTAAATTAGTTTGGTTGGATGCAG
Gp25-XmaI	ATTTTC**CCCGGG**CTTCTCCTTTACCGTTACATATTTA
Gp53-NcoI	TGGCGA**CCATGG**AAGCAAAATTAACTAAAAATGAGTTT
Gp53-XmaI	TATGTC**CCCGGG**ACTGATTTCTCCCCATAAGTCA
lysSPP1Fw	CC**GGTACCACTAGT**AATGAAGGAGAGTGAAAGAATATGAGTAA
pIV23d-His-Eco	CGCCAGTGTGCTG**GAATTC**G
SPbprFw	TAAAAA**ACTAGT**TGAAAAAGGGGGATGAAAATGAG
SPbprRv	TGCCCCGGCTGCTCCCGGA
SPbprLYSfw	CTGCTGTTTCCGGGAGCAGCCGGGGCAAGTAAATTAGTTTGGTTGGATGCAGGTCATG
Pxyl-Mfe	GAA**CAATTG**ATCTCTGCAGTCGCGATGATTA
Pxyl-Kpn	CGG**GGTACC**GGATCCCATTTCCCCCTTTGA
gp24.1SPP1fw	AA**GGTACCGTCGAC**TAAAAGGGGGATGAAGAAAATGCCGGA
gp24.1-holSPP1rev2	TTAGTTCTTTACAAGGTTCCATACA
gp24.1-holSPP1fw	ACAATTGTATGGAACCTTGTAAAGAACTAACGGTAAAGGAGAAGTAAAATGAAAATGG ATACA
holSPP1rv	TGGC**ATCGAT**CTTCGTCAATCCGTTTTTCTTCAATG
HAcMycrv	TT**GCATGCCCGCGG**GCTTGCATGGAAAAAAGCCCGCTCA
lysSPP1fwSacI	AGTGA**GAGCTC**ATGAGTAAATTAGTTTGGTTGGATG
pIV23d-His-Sal	CCA**GTCGAC**TGGAATTCGCCCTTTTATTAATGA
HolSPP1fwSal	ATG**GTCGAC**TAAAGGAGAAGTAAAATGAAAATGGA
gp27rvNsi	GCTACT**ATGCAT**ATGGAATAACCGTCAACCGGGT
lysSPP1rv	TTTC**ATCGAT**CTTCTCCTTTACCGTTACATATTTACT

[1]Restriction sites are in bold case and underlined: CCATGG, *Nco*I; CCCGGG, *Xma*I; GGTACC, *Kpn*I; ACTAGT, *Spe*I; GAATTC, *Eco*RI; CAATTG, *Mfe*I; GTCGAC, *Sal*I; ATCGAT, *Cla*I; GCATGC, *Sph*I; CCGCGG, *Sac*II; GAGCTC, *Sac*I; ATGCAT, *Nsi*I.

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CHAPTER 4.
PROBING THE FUNCTION OF THE TWO HOLIN-LIKE PROTEINS OF BACTERIOPHAGE SPP1.

This chapter contains data submited in:

- Fernandes, S. and São-José, C. (2016) Probing the function of the two holin-like proteins of bacteriophage SPP1. Submitted to *Virology*.

Probing the function of the two holin-like proteins of bacteriophage SPP1

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Running title: **Phage SPP1 holin**

Key words: holin; SPP1; *B. subtilis*; endolysin; promoter; bacteriophage; lysis

Virology

(2016, submitted)

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Abstract

Double-stranded DNA bacteriophages employ holin and endolysin functions to lyse host bacteria after virus multiplication. Holins oligomerize in the cytoplasmic membrane and trigger to form holes that cause cell death. For most systems these holes are also required for endolysin release to the cell wall, where it cleaves the peptidoglycan network. *Orfs 25* and *26* of *Bacillus subtilis* phage SPP1 were predicted to encode the endolysin and holin functions, respectively. However, the product of the upstream *orf 24.1* exhibits also holin features. We show that production of gp24.1 or gp26 in *B. subtilis* causes no major impact on cell growth, despite their ability to insert in the cytoplasmic membrane. Instant growth cessation and cell death is observed only upon co-production of the two holin-like proteins. Surprisingly, a constitutive promoter was identified within *orf 24.1*, which we propose to correspond to the previously described SPP1 early promoter *P*E5.

4.1 Introduction

The holin and endolysin lytic functions seem to be universal players in the strategies used by double-stranded DNA phages to lyse bacterial hosts and to allow escape of the viral progeny (for reviews on holin and endolysin features and mode of action see Catalão *et al*., 2013; Young, 2013, 2014). Endolysins are enzymes that cleave the cell wall (CW) peptidoglycan, whereas holins are proteins with one or more transmembrane domains (TMDs) that insert and accumulate in the cytoplasmic membrane (CM). At a genetically-defined time holins suddenly induce the formation of holes, which lead to collapse of the CM proton motive force (pmf) and instant cell death (the holin lethal effect). For phages following the so-called canonical lysis model, of which phage λ is the best studied example, the holin holes are crucial for lysis of infected cells as they provide a channel for passage of the cytosol-accumulated endolysin to the CW (the holin transport effect, Savva *et al*,. 2014). In non-canonical systems the endolysins are typically exported to the CW by holin-independent mechanisms. In these cases the key holin role is its lethal effect, which is essential to liberate endolysins from activity-restraining mechanisms that act on the exported enzymes (for details see Catalão *et al*., 2013; Frias *et al*., 2013; Young, 2013, 2014). The pmf-dissipating action of the holin can also be important to potentiate the lytic action of canonical endolysins after reaching the CW (Fernandes and São-José, 2016).

Holins constitute a very diverse functional group. Frequently they are small hydrophobic proteins (<150 aa) with at least 1 TMD and with a hydrophilic and highly charged C-terminus (Wang *et al*., 2000; Young, 2002). Holins oligomerize within the CM of the bacterium and, as long as the pmf is maintained above a certain threshold, they assemble into oligomers and rafts of intrinsic stability. Below this threshold holins trigger for hole formation (Wang *et al*., 2000; Young *et al*., 2000; Young and Wang, 2006). From a mechanistic point of view holins can be divided in canonical holins and pinholins. As described above, in canonical lysis the holin makes pores large enough to allow the translocation of the endolysin from the cytoplasm to the CW (Young, 2013). Pinholin is the term used to describe holins that form small-holes that are meant for passage of small ions and CM depolarization (Park *et al*., 2007). Pinholins are typically associated to phages that produce exported endolysins (Catalão *et al*., 2013; Young, 2013, 2014).

From the above it is easily concluded that the holin action needs to be delayed during the initial stages of phage infection to avoid premature cell death and/or lysis. In addition to mechanisms controlling holin transcription and translation (Catalão *et al*., 2013), phages can employ a holin antagonist, the antiholin, for the fine tuning of lysis timing. The antiholin mode of action is best studied in lambdoid phages and in phage T4 (Young, 2013, 2014 and references therein). In lambdoid phages the holin gene itself, through a dual-start motif encodes both the lysis and the inhibitor functions (Bläsi and Young, 1996), whereas in T4 these functions are encoded by separate genes (Ramanculov and Young 2001). The antiholin exerts its function by interacting and/or by oligomerizing with the holin, but after pmf-collapse both can contribute to hole formation (Gründling *et al*., 2000; Tran *et al*., 2005).

Interestingly, it was reported for at least two phages that well-timed and efficient lysis required the concerted action of two holin-like proteins produced from two separate genes in the lysis cassette. These are the proteins Gp4 and Gp5 of mycobacteriophage Ms6 (Catalão *et al*., 2011) and XhlA and XhlB of the *B. subtilis* defective prophage PBSX (Krogh *et al*., 1998). It was proposed for both phages that the two holin-like proteins interact to form the holin functional unit responsible for efficient endolysin release at the proper timing for lysis (Catalão *et al*., 2011; Krogh *et al*., 1998).

We showed recently that the PBSX lytic gene cluster shares some features with the putative lysis cassette of the *B. subtilis* phage SPP1 (Fernandes and São-José, 2016). Previous genome sequence analysis assigned *orfs 25* and *26* (Fig. 4.1) as the endolysin and holin genes of phage SPP1, respectively (Alonso *et al*., 1997). We have confirmed the endolysin function of gp25 (renamed LysSPP1) and have highlighted the sequence and structural relatedness between XhlB and gp26 and between XhlA and gp24.1 (Fernandes and São-José, 2016). The latter is the product of *orf 24.1*, which lies immediately upstream of gene *25* in SPP1 (Fig. 4.1).

In agreement with their holin-like features, preliminary work showed that co-expression of *orfs 24.1* and *26* in *B. subtilis* resulted in rapid loss of cell viability (Fernandes and São-José, 2016). In the present study we performed a functional characterization of gp24.1 and gp26, assessing the contribution of each protein to the cell killing effect. The results indicate that the typical lethal character of the holin action is only observed when

gp24.1 and gp26 are simultaneously produced. We reveal also the presence of a constitutive promoter within *orf 24.1* and discuss its possible role in SPP1 lysis regulation.

4.2 Results

4.2.1 Impact of *24.1* and *26* expression in *B. subtilis* cells

As mentioned above, one paramount feature of holin activity is its capacity to insert in the host cell CM and form lethal holes (Young, 2013). In order to gain insight on the putative holin function of SPP1 gp24.1 and gp26, the corresponding *orfs* were expressed in *B. subtilis*, either individually or as a transcriptional fusion, and the effect on cell growth and viability evaluated. This was achieved by cloning *24.1*, *26* and the *24.1-26* fusion in a replicative plasmid under control of a xylose-inducible promoter (plasmid pNPxyl). The constructs allowed production of gp24.1 and gp26 C-terminally fused to the c-Myc tag when the corresponding *orfs* were individually expressed. When co-synthesized from the transcriptional fusion the tag was only added to gp26 (see materials and methods). In the three constructs the native translation signals of *24.1* and *26* were preserved, including an in frame alternative start codon and ribosome binding site that we have identified upstream of the annotated translation signals of *24.1* (Fig. 4.1).

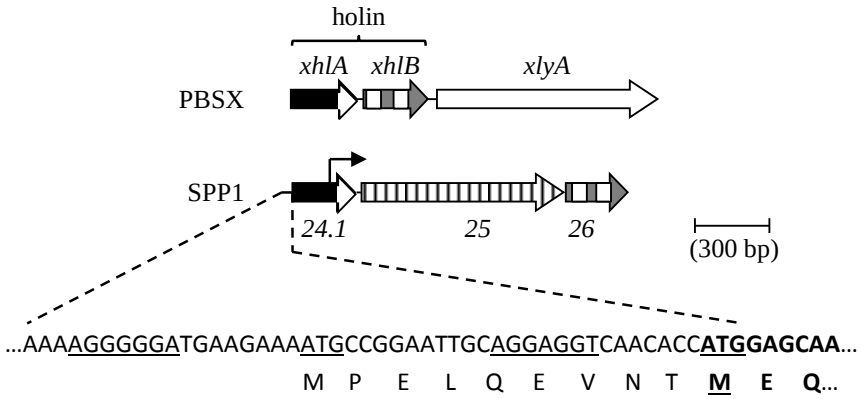

Fig. 4.1. Lysis genes of *B. subtilis* phages SPP1 and PBSX. Representation of the genome segments of phages SPP1 (Acc. N0. X97918) and PBSX (Acc. N0. NC_000964) encompassing the endolysin (white and stripped arrows) and holin-like (black and gray arrows) genes. Homology between deduced gene products is highlighted by the same filling pattern. XhlA and gp24.1 are members of the Pfam family XhlA (pfam10779), which includes cell-surface associated haemolysins. XhlB and gp26 are members of the Pfam family Phage_holin (pfam04688), which includes several holin-like proteins from Siphovirus phages. The white regions in the holin-like genes indicate the number and relative position of putative transmembrane domain coding sequences, according to the TMHMM tool (http://www.cbs.dtu.dk/services/TMHMM). Endolysins XlyA and gp25 (LysSPP1) display catalytic domains of the families Amidase_2 and Amidase_3, respectively, as defined by Pfam analysis (http://pfam.xfam.org). The bent arrow indicates the presence of promoter activity within *24.1* (see text). Sequence details (from nucleotide positions 20526 to 20579 of Acc. N0. X97918) of the two putative translation start signals of *24.1* are shown below, with indication of the deduced N-terminal amino acid sequence (the annotated start in sequence databases is depicted in bold/underlined).

In absence of the inducer growth of *B. subtilis* cells carrying either *24.1* or *26* was indistinguishable from that of cells carrying the empty pNPxyl. In contrast, the strain carrying the *24.1-26* fusion presented delayed growth in the same conditions, suggesting some toxicity derived from basal expression of the putative holin-like genes (Fig. 4.2A). Induction of half of the cultures with xylose at mid-exponential phase did not affect growth of cells harboring either of the holin-like genes, except that cells expressing *orf 26* appeared to reach slightly lower optical densities (OD_{600nm}) values when entering stationary growth (Fig. 4.2A). Quite oppositely, induction of the cultures carrying *24.1-*

26 resulted in instant growth interruption, which was accompanied by a drastic decrease of cell viability (2-log reduction of cfu counts) (Fig. 4.2A).

The production of gp24.1 and gp26 upon xylose induction was accessed by western blot analysis using anti-c-Myc antibodies (Fig. 4.2B and C). Note that in the strain used to co-synthesize gp24.1 and gp26 only the latter is tagged with c-Myc. As observed in Fig. 4.2C, when produced separately gp24.1 and gp26 were only detected after addition of the inducer, with gp24.1 apparently accumulating in larger amounts than gp26. In some western blots gp24.1 seemed to be detected as a band doublet (see below). Surprisingly, gp26 was detected in the extracts of non-induced cultures of the *24.1-26* strain at levels comparable to those of the induced *26* strain (Fig. 4.2C). The gp26 signal further increased after xylose addition to the *24.1-26* cultures. Since we did not find any mutation in the construct of the transcriptional fusion *24.1-26* that could justify the production of gp26 in absence of xylose, we raised the hypothesis that constitutive promoter sequences within *orf 24.1* could be driving the expression of *orf 26* (see next).

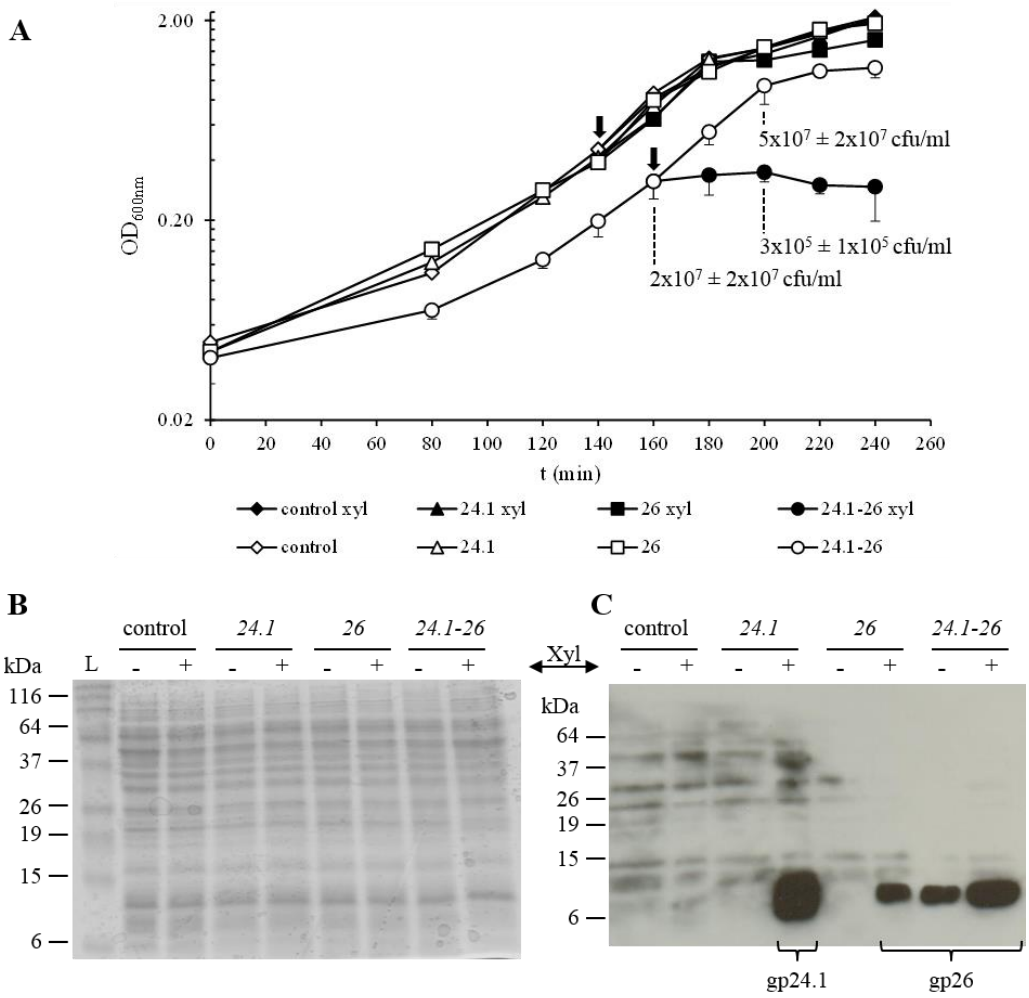

Fig. 4.2. Expression of *orfs 24.1* and *26* in *B. subtilis* and effect on cell growth. (A) Growth curves of *B. subtilis* cells expressing *orfs 24.1*, *26* or co-expressing both (*24.1-26*). The "control" curves correspond to cells carrying the empty expression vector pNPxyl. Expression was induced in half of the cultures (filled symbols) at the indicated time points (black arrows) by adding 0.5 % xylose (xyl). Cell viability (cfu/ml) of cells co-expressing *24.1* and *26* was scored at the indicated time points in absence or presence of xylose. **(B)** Coomassie blue-stained SDS-PAGE gel of total protein extracts produced from the cultures described in panel A at time points t = 180 min (control, *24.1* and *26*) and t = 200 min (*24.1-26*), in absence or presence of xylose. The molecular weight of bands composing the protein ladder (L) is indicated on the left side. **(C)** Detection by western blot analysis of recombinant gp24.1 and gp26 in the protein extracts described in panel B, using anti-c-Myc specific antibodies. Note the production of gp26 in absence of xylose in cells carrying the *24.1-26* fusion.

4.2.2 *Orf 24.1* carries internal promoter sequences that are functional both in *E. coli* and *B. subtilis*

To test the presence of transcription promoter sequences within *24.1*, the naturally adjacent *orfs 24.1* and *25* (Fig. 4.1) were cloned in the promoter-less shuttle vector pNW33N, with the 3' end of the endolysin gene fused to a hexahistidine-coding sequence for protein detection. Endolysin production served thus as reporter of promoter activity located upstream gene *25*. For control purposes we constructed a pNW33N derivative carrying the *25His* fusion only. *E. coli* and *B. subtilis* strains individually carrying each of the constructs were generated.

As shown in Fig. 4.3A and B, a strong gp25 (LysSPP1) signal was observed only when the endolysin gene was preceded by *orf 24.1*, both in *E. coli* and *B. subtilis*. The results strongly suggested that the *24.1* sequence carried a constitutive promoter active during bacterial vegetative growth, that is, a σ^A- and σ^{70}-dependent promoter in *B. subtilis* and *E. coli*, respectively (Haldenwang, 1995). In fact, analysis of the *24.1* sequence in parallel with that of known SPP1 early promoters (σ^A-dependent *P*E1 to *P*E3, Alonso *et al*., 1997; Pedré *et al*., 1994) revealed the -35 and -10 elements of a putative promoter (Fig. 4.3C). The -10 box displayed the "TG extension" typical of strong promoters (Helmann, 1995). In conclusion, the results indicated that *24.1* carries an internal σ^A-dependent promoter that, in absence of xylose, was responsible for the constitutive production of gp26 by *B. subtilis* cells carrying the *24.1-26* fusion (Fig. 4.2). The nature and possible implications of this promoter in lysis regulation during SPP1 infection are discussed below.

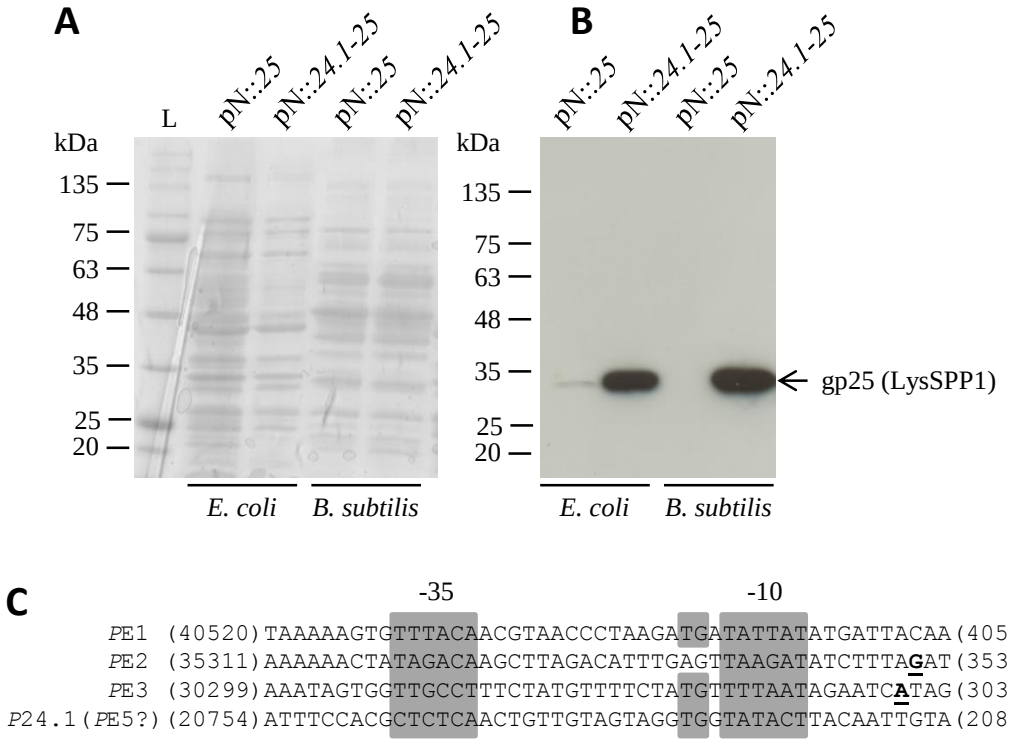

Fig. 4.3. Analysis of promoter activity within *orf 24.1*. (A) Coomassie blue-stained SDS-PAGE gel of total protein extracts produced from *E. coli* and *B. subtilis* cells carrying either gene *25* or the natural *24.1-25* arrangement (Fig. 4.1) cloned in the promoter-less, shuttle vector pNW33N. In the constructs the 3' end of gene *25* was fused to a His_6 coding sequence. The molecular weight of bands composing the protein ladder (L) is indicated on the left side. **(B)** Detection by western blot analysis of recombinant gp25 in the protein extracts described in panel A, using anti-His_6 specific antibodies. The endolysin gp25 is detected as an intense band only when the *24.1* sequence is present upstream gene *25*. **(C)** Alignment of the nucleotide sequences of the previously identified SPP1 early promoters *P*E1, *P*E2 and *P*E3 with that of the putative promoter detected within *24.1*, which we propose to correspond to *P*E5 (see text). Sequence coordinates are according to the SPP1 genome sequence (Acc. N0. X97918). The elements -35 and -10 and the "TG extension" are shadowed (putative in *24.1*). Known transcription starts sites (+1) are bold/underlined (Pedré *et al*., 1994).

4.2.3 The holin-like proteins gp24.1 and gp26 localize to the cytoplasmic membrane

Holins, like that of phage λ have been shown to insert and accumulate in the CM until they trigger for hole formation (Gründling *et al*., 2001). We have studied how gp24.1 and gp26 distributed between the cytosol and the CM when independently produced in *B.*

subtilis. The distribution of gp26 was also accessed when co-synthesized with gp24.1. In all situations the holin-like products were either exclusively detected or detected in much larger amounts in the CM fraction (Fig. 4.4), clearly indicating that these proteins are targeted to this cell compartment.

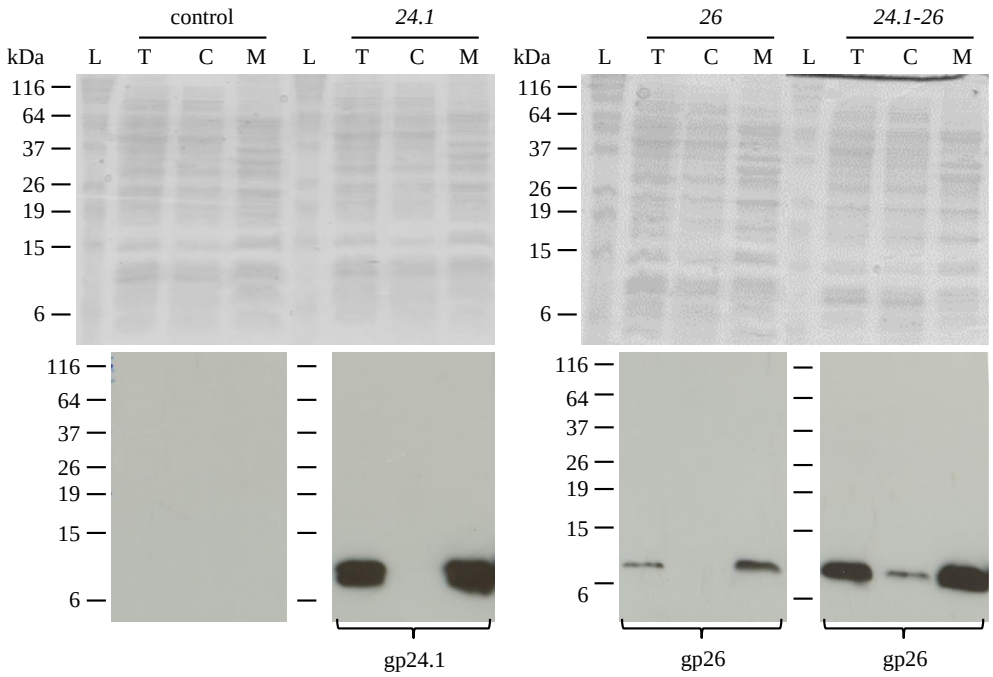

Fig. 4.4. Membrane localization of gp24.1 and gp26. Xylose-induced cells of *B. subtilis* strains expressing *orfs 24.1*, *26* or co-expressing both (*24.1-26*) were fractionated into total (T), cytoplasmic (C) and membrane (M) protein extracts and analyzed by SDS-PAGE/Coomassie blue staining (top panels) and by western blot with anti-c-Myc specific antibodies (bottom panels). The molecular weight and position of bands composing the protein ladder (L) is indicated as in the previous figures.

4.3 Discussion

Probably due to the lack of suitable tools to control expression of their lethal character, holins from phages infecting Gram-positive bacteria have been almost exclusively studied in heterologous systems, mostly in *E. coli*. Frequently, putative holin genes are expressed from a plasmid vector, alone or in combination with their endolysin counterparts, and the effect on *E. coli* viability and cell lysis evaluated. In another approach, the holin function of a giving protein has been assumed based on its capacity to complement the lysis-defective phenotype of mutant λ phages affected in holin synthesis (Catalão *et al*., 2011;

Díaz *et al*., 1996; Martín *et al*., 1998; São-José *et al*., 2004; Vukov *et al*., 2000). A still open question though is if the results obtained in heterologous hosts can be easily transposed to the native systems.

As referred to above, there are very few examples in the literature of vector-mediated holin expression in natural Gram-positive hosts (Martín *et al*., 1998). In some cases however, it has been possible to inactivate putative holin genes in prophages or phages and confirm the holin function when host cell lysis is affected. This was done for the holin-like proteins XhlA and XhlB of PBSX, Gp4 and Gp5 of mycobacteriophage Ms6 and Svh1 and Svh2 of pneumococcal phage SV1 (Catalão *et al*., 2011; Frias *et al*., 2013; Krogh *et al*., 1998).

In this work we have studied how expression of the SPP1 holin-like genes *24.1* and *26* affected host cells growth and viability, with the goal of understanding their contribution to the holin function. We show that despite the capacity of gp24.1 and gp26 to insert in the CM when individually produced, growth interruption and cell death typical of holin activity seems to depend on the simultaneous expression of the two holin-like genes. This suggests that the SPP1 holin function requires the synthesis and probably the interaction of these two proteins, as suggested or demonstrated for the holin-like pairs in PBSX and Ms6. However, it is currently unknown how the production of gp24.1 and gp26 in our expression conditions compare to those occurring in the natural context of SPP1 infection. In addition, gp24.1 and gp26 appeared to accumulate at different levels, especially when produced separately, with gp24.1 being consistently detected with stronger signals than gp26 (Fig 4.2C). Therefore, one cannot totally exclude that higher levels of gp26 accumulation would not result in increased cell toxicity. Still, we believe that the relative lower production of gp26 by induced cultures expressing *orf 26* only (Fig. 4.2A) should not explain its poor effect on cell growth. In fact, because of the promoter sequences within *24.1*, similar gp26 amounts were detected in non-induced cultures of cells co-expressing *orfs 24.1-26* (Fig. 4.2B and C), but in this case with a clear impact on cell growth rate (Fig. 4.2A). Our interpretation is that the delayed growth of the *24.1-26* strain in absence of the inducer probably results from a basal, undetectable level of gp24.1 production, which in combination with gp26 induces some toxicity to the cell population.

An unexpected finding was the presence of a constitutive, σ^A-dependent promoter internal to *orf 24.1*. Given its localization and apparent strength it is most likely that this promoter corresponds to the previously described SPP1 early promoter *P*E5 (Alonso *et al*., 1997; Montenegro and Trautner, 1981; Stüber *et al*., 1981). *P*E5, whose activity starts to be detected up to 5 min after the beginning of SPP1 infection, was tentatively mapped upstream *orf 27* (Alonso *et al*., 1997; Montenegro and Trautner, 1981). We see this promoter activity initiated within *orf 24.1* as indirect evidence supporting that the SPP1 holin function should not rely exclusively on the gp26 action. In fact, its occurrence implies that the downstream endolysin and holin-like genes *25* and *26*, respectively, belong to the class of genes that are early transcribed during *B. subtilis* infection by SPP1. Therefore, it is expected that LysSPP1 and gp26 start to be produced at early stages of phage multiplication. If gp26 was the sole protein responsible for the holin function it could result in premature host cell lysis.

Gp24.1 could harmlessly accumulate at high levels in the *B. subtilis* CM. Curiously, of the two holin-like proteins of PBSX, XhlA (the gp24.1 homologue) was the one that seemed to cause cell damage when expressed in *B. subtilis* (Krogh *et al*., 1998). To discard the possibility that the C-terminal c-Myc was interfering with gp24.1 activity, we have similarly expressed in *B. subtilis* an untagged version of the protein. As observed for the tagged gp24.1, its production caused no effect on cell growth (data not shown). The holin-like gene *24.1* seems to carry two in frame alternative translation starts (Fig. 4.1), which might explain the frequent detection of gp24.1 as a band doublet (Fig. 4.4). In *xhlA* of PBSX and PBSZ (the defective prophage in *B. subtilis* strain W23) there is also a putative ribosome binding site and ATG start cordon upstream that annotated in databases, but these are out of frame and interrupted by a stop codon (Fig. S4.1). Wether this is on the basis of the different gp24.1 and XhlA phenotypes when expressed in *B. subtilis* is currently unknown. The vast majority of the closest gp24.1 homologues are annotated as starting by a methionine equivalent to that of the first *24.1* start codon, but some exhibit also potential alternative start sites (not shown). Alternative translation initiation seems thus to be a common feature of holin and holin-like genes, as well exemplified by the phage λ *S* gene and the *Clostridium difficile tcdE*, which is involved in toxin release (Bläsi and Young, 1996; Govind *et al*., 2015).

One key role of holins in canonical lysis is to form lesions in the CM large enough for endolysin escape to the CW. We have tried to produced LysSPP1 from a IPTG-inducible promoter (Fernandes and São-José, 2016) in cells expressing *24.1-25*, with the goal of demonstrating the role of gp24.1 and gp26 in endolysin release to the CW. Unfortunately, probably because of leaky transcription in absence of the inducers we were unable to stably maintain the constructs expressing the three lysis genes within the same *B. subtilis* cell.

In conclusion, considering the apparent requirement of gp24.1 and gp26 for holin lethality and the occurrence of a promoter inside *orf 24.1* (most likely the SPP1 early promoter *P*E5), our hypothesis is that a steady state of LysSPP1 and gp26 production is achieved during early phage development without compromising the infected cell. Activation of late transcription from the upstream promoters *P*L3 to *P*L5, which peaks between 12 and 30 min after infection (Alonso *et al*., 1997; Montenegro and Trautner, 1981), would result in the gradual accumulation of gp24.1 and in further increase of LysSPP1 and gp26 production, ultimately leading to cell lysis. This tentative model for the regulation of SPP1-mediated host cell lysis is compatible with the transcription profile of the phage genome (Alonso *et al*., 1997; Montenegro and Trautner, 1981; Stüber *et al*., 1981).

4.4 Materials and methods

4.4.1 Biological material, growth conditions and general techniques

Bacterial strains, phages, plasmids and primers used in this study are indicated in Table S4.1. *E. coli* and *B. subtilis* strains were routinely pre-cultured overnight at 37ºC and 30ºC, respectively, in LB medium (Sambrook and Russel, 2001) under orbital shaking. New cultures were initiated by diluting pre-cultures 100-fold in fresh medium. Bacterial growth was monitored by taking OD_{600nm} measurements at regular intervals. LB was supplemented with 0.01% glucose when growing *B. subtilis* strains carrying plasmid pNPxyl or its derivatives (see below) to minimize basal expression from the P_{xylA} promoter. For selection of strains carrying vectors or recombinant plasmids LB was supplemented with 100 μg/ml ampicillin or 20 μg/ml chloramphenicol (for *E. coli*) and 5 μg/ml chloramphenicol (for *B. subtilis*). SPP1 phages were propagated in *B. subtilis* strain YB886 as described previously (Jakutytè *et al*., 2011).

Plasmids were constructed following standard DNA techniques (Sambrook and Russel, 2001). Phage DNA was isolated as described in Vinga *et al*. (2012). High-fidelity polymerase chain reaction (PCR) was carried out with Kod Hot Start Master Mix (Novagen, USA). Transformation of *E. coli* and *B. subtilis* strains followed the protocols described by Chung *et al*. (1989) and Yasbin *et al*. (1975), respectively. Constructs were confirmed by DNA sequencing (GATC Biotech).

Two or 4 ml-samples of *B. subtilis* cultures were used for routine production of total protein extracts as described previously (Fernandes and São-José, 2016). Protein extracts were quantified with Bradford reagent (Bio-Rad Laboratories), using bovine serum albumin as standard. Approximately 20 ug of total protein were separated by SDS-PAGE (15% resolving gels for holin-like proteins and 11% for LysSPP1) and proteins analyzed by western blotting as described elsewhere (Sambrook and Russell, 2001). His_6- and c-Myc-tagged proteins were immunodetected with 0.4 μg/ml of anti-His_6 and 1μg/ml of anti-c-Myc antibodies, respectively, according to manufacturer instructions (Roche Applied Science), and with antigen/antibody complexes being revealed with the Luminata Forte Western HRP Substrate (Merck Millipore).

4.4.2 Plasmid constructions

The putative holin-like genes *24.1* and *26* were PCR-amplified with the primer pairs gp24.1SPP1fw/gp24.1SPP1rv1 and gp26SPP1fw/gp26SPP1rv, respectively. The resulting products were digested with *Kpn*I and *Cla*I and ligated to the equally digested pMutin-cMyc (Kaltwasser *et al*., 2002), resulting in the translation fusion of the 3' end of each gene to the coding sequence of the c-Myc epitope carried in pMutin-cMyc. The DNA segments *24.1cMyc* and *26cMyc* were then PCR-amplified from the ligation reactions using the primer pairs gp24.1SPP1fw/HAcMycrv and gp26SPP1fw/HAcMycrv, respectively, the products were cleaved with *Kpn*I and *Sph*I and finally inserted into the similarly digested pNPxyl, yielding plasmids pNP::24.1cMyc and pNP::26cMyc. In these plasmids genes *24.1cMyc* and *26cMyc* are under the transcriptional control of the *xylR*-P_{xylA} cassette, being their expression induced in presence of xylose (Fernandes and São-José, 2016). Plasmid pNP::24.1-26cMyc, which allows the xylose-inducible expression of the transcriptional fusion *24.1-26cMyc* was constructed previously by following the same strategy (Fernandes and São-José., 2016).

Plasmid pNP::24.1cMyc was obtained in *E. coli* strain TG1 and then used to transform *B. subtilis* YB886, yielding the strain YB/pNP::24.1cMyc. Plasmid pNP::26cMyc was obtained directly in YB886, yielding strain YB/pNP::26cMyc. The YB886 recombinant strains carrying the described pNPxyl derivatives were selected in the presence of 0.1% of glucose for catabolite repression of promoter P_{xylA}.

The expression of gene *25* (encodes the SPP1 endolysin LysSPP1), when cloned downstream of *orf 24.1*, was used to confirm the presence of promoter sequences internal to the latter putative gene. For that, the region *24.1-25His* was amplified from the mutant phage SPP1*g25His* (Fernandes and São-José., 2016) with the primer pair gp24.1SPP1fw/pIV23d-His-Sal. The PCR product was cleaved with *Sal*I and ligated to similarly digested pBluescript II KS (+). The resulting plasmid pKS::24.1-25His was then double-digested with *Xba*I/*Kpn*I and the released *24.1-25His* DNA segment inserted into the equally digested, promoter-less *E. coli*/*B. subtilis* shuttle vector pNW33N, yielding plasmid pNW::24.1-25His. As control we constructed an analogous plasmid (pNW::25His) carrying only gene *25His*, following the same strategy described above and using the previously constructed plasmid pKS::25His (Fernandes and São-José., 2016) as template. Both pNW33N derivatives were first isolated in *E. coli* XLIBlue-MRF' and then used to transform *B. subtilis* YB886, yielding strains YB/pNW24.1-25His and YB/pNW25His.

4.4.3 *B. subtilis* cell fractioning

Five hundred milliliter cultures of *B. subtilis* strains harboring pNPxyl or its derivatives were grown until OD_{600nm} = 0.3-0.4 (for YB/pNP, YB/pNP::24.1cMyc and YB/pNP::26cMyc) or OD_{600nm} = 0.6-0.7 (for YB/pNP::24.1-26cMyc) and then induced with 0.5% xylose. Cells were collected at OD_{600nm} 0.8-1.0 and resuspended in 10 ml lysis buffer (20mM Hepes, 300mM NaCl) supplemented with 100 µg/ml lysozyme and 1X protease inhibitor cocktail (Complete EDTA-free Protease Inhibitor Cocktail; Roche Applied Science). From this point on, cell disruption and production of total, cytoplasm and membrane fractions were as described in Jakutytè *et al*. (2011).

4.4.4 Bioinformatic analysis

The tools used for analysis of protein homologies and for identification of conserved domains and putative transmembrane helices were those described in Fernandes and São-José., 2016.

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4.6 Supplementary materials

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>24.1 SPP1
CAATAAAAGGGGGATGAAGAAAATGCCGGAATTGCAGGAGGTCAACACCATGGAGCAA
                      M  P  E  L  Q  E  V  N  T  M  E  Q

>xhlA1 B. amyloliquefaciens
AGATAAAAGGGGGGCGTACTAATGTCACAAATGACGGAGGTACCGGAAGTGAATGCT
                     M  S  Q  M  T  E  V  P  E  V  N  A

>xhlA PBSX
CCTCGGAAGGGAGGTGATCTGCATGTGAAGGAGGAGTGAGAGATGCAGCAAGAG
                                          M  Q  Q  E

>zhlA PBSZ
CCTCGGAAGGGAGGTGATCCGCATGTAAAGGAGGAGTGAGTAATGCAGCAAGA
                                          M  Q  Q  E
```

Fig. S4.1. **Putative translation signals in *24.1*/*xhlA*-like genes.** Putative ribosome binding sites and start codons are in bold and underlined. Annotated starts in databases are shaded in yellow whereas stop codons are in red. SPP1 (Acc. N0. X97918); *Bacillus amyloliquefaciens* DSM7 (Acc. N0. FN597644); PBSX (Acc. N0. NC_000964); PBSZ (Acc. N0. ADM37359).

Table S4.1. Bacterial Strains, Phages, Plasmids and Primers Used in This Study

Bacteria, bacteriophages, plasmids and primers		
	Genotype and/or relevant features	**Reference[1]/ Source**
		***E. coli* strains**
XLIBlue-MRF'	*Δ*(*mcrA*)*183 Δ*(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI*q *lacZΔM15* Tn*10* (Tetr)]; cloning host.	Stratagene
TG1	*supE thi-1 Δ*(*lac-proAB*) *Δ*(*mcrB-hsdSM*)*5*, (*rK-mK-*) [F' *traD36 proAB+ lacIq lacZΔM15*]; cloning host.	Stratagene
		***B. subtilis* strains**
YB886	*B. subtilis* 168 derivative free of prophages PBSX and SPβ; SPP1 indicator strain	4
YB/pNP::24.1cMyc	YB886 derivative harboring pNP::24.1cMyc	This work
YB/pNP::26cMyc	YB886 derivative harboring pNP::26cMyc	This work
YB/pNP::24.1-26cMyc	YB886 derivative harboring pNP::24.1-26cMyc	1
YB/pNP	YB886 derivative harboring pNPxyl	1
YB/pNW::24.1-25His	YB886 derivative harboring pNW::24.1-25His	This work

Table S4.1 (continued)

YB/pNW::25His	YB886 derivative harboring pNW::25His	This work
		Bacteriophages
SPP1	*B. subtilis* strictly lytic phage	3
SPP1g25His	SPP1 derivative carrying the 3' end of endolysin gene *25* fused to a His_6-tag sequence	1
Plasmids		
pBluescript II KS(+) (pKS)	*E. coli* cloning vector; Amp^r	Stratagene
pNW33N	*E. coli/B. subtilis* shuttle vector; Cm^r	Mee and Welker, unpublished/ BGSC[2]
pMutin-cMyc	*B. subtilis* integration vector; source of c-Myc epitope	2/ BGSC[2]
pNPxyl	Expression vector; pNW33N derivative carrying the expression cassette *t0-xylR-PxylA*	1
pNP::24.1cMyc	pNPxyl derivative carrying the SPP1 holin-like gene *24.1*	This work
pNP::26cMyc	pNPxyl derivative carrying the SPP1 holin-like gene *26*	This work
pNP::24.1-26cMyc	pNPxyl derivative carrying a transcriptional fusion of the SPP1 holin-like genes *24.1* and *26*	1
pKS::25His	pKS derivative carrying gene *25* fused to a His_6-tag	1
pKS::24.1-25His	pKS derivative carrying the *24.1-25His* DNA segment from SPP1*g25His*	This work
pNW::25His	pNW33N derivative carrying gene *25His* from pKS::25His	This work
pNW::24.1-25His	pNW33N derivative carrying the *24.1-25His* DNA segment from pKS::24.1-25His	This work
		Primers
	Sequence (5' → 3')[3]	
gp24.1SPP1fw	AA**GGTACCGTCGAC**TAAAAGGGGGATGAAGAAAATGCCGGA	
gp24.1SPP1rv1	TCCT**ATCGAT**GTTCTTTACAAGGTTCCATACAATTGT	
gp26SPP1fw	AA**GGTACCGTCGAC**CGGTAAAGGAGAAGTAAAATGAAAATGGA	
gp26SPP1rv	TGGC**ATCGAT**CTTCGTCAATCCGTTTTTCTTCAATG	
HAcMycrv	TT**GCATGCCCGCGG**GCTTGCATGGAAAAAAGCCCGCTCA	
pIV23d-His-Sal	CCA**GTCGAC**TGGAATTCGCCCTTTTATTAATGA	

[1]The references corresponding to the numbers indicated in the Table can be found in section 4.6.1, [2]Bacillus Genetic Stock Center, Ohio State University, USA.

[3]Restriction sites are in bold case and underlined: GGTACC, *Kpn*I; GTCGAC, *Sal*I; ATCGAT, *Cla*I; GCATGC, *Sph*I; CCGCGG, *Sac*II

4.6.1 References of supplementary materials

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2- Kaltwasser, M., Wiegert, T., and Schumann, W. (2002). Construction and application of epitope- and green fluorescent protein-tagging integration vectors for *Bacillus subtilis*. *Appl Environ Microbiol* **68**: 2624-2628.

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CHAPTER 5.
CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The golden era of antibiotic discovery occurred in the 1950s-1970s and very few new classes of these agents have been discovered since then. This, associated with the rise of antibiotic resistance among important pathogenic bacteria motivated the need to considerer new antibacterial approaches. A recent survey of the antibiotic alternatives pipeline has included endolysins amongst the agents with greatest potential, although the most optimistic projections indicate that they should not reach the registration phase of clinical development before 2022 (Czaplewski *et al*., 2016). Endolysins mode of action differs from that of antibiotics and therefore the lytic enzymes are capable of killing antibiotic resistant bacteria. In addition, bacterial resistance to endolysins has not been reported to date. There are nevertheless some potential limitations regarding the therapeutic application of endolysins, notably host immune response and enzyme inactivation by antibodies. The typical narrow spectrum of activity of endolysins and the high costs associated with their production may also be additional obstacles.

Something that seems to emerge from the most recent literature is that in order to consider endolysins as a real alternative to antibiotics, probably their study needs to go beyond the simple identification and purification of native endolysins. Protein engineering may be required for large scale production of effective lytic enzymes and a better understanding of the factors influencing their activity in different environments is also needed. This doctoral project was developed in line with these ideas. From the one hand, we have demonstrated that the construction of chimeric endolysins using domains of heterologous origin can result in enzymes with improved features. On the other hand, we have shown that dissipation of the bacterial pmf, which occurs in the last stages of phage infection by the holin action, may be a general requirement for endolysins to display full lytic activity. We think these findings should be taken into consideration in the enzybiotics field, as it may be necessary to select or engineer endolysins that can bypass this holin sensitization requirement for maximal killing efficacy.

We showed in the first part of this study that the construction of chimerical endolysins, combined with gene expression at low temperatures, can be a good strategy for the efficient production of soluble endolysins in *E. coli*. By following this approach we obtained recombinant enzymes displaying broad lytic activity *in vitro* against clinically relevant strains of *S. aureus*, including MRSA (Chapter 2). Heterologous overproduction and subsequent purification of endolysins frequently results in low yields of soluble

proteins, with reduced lytic efficacy. Additionally, most native endolysins exhibit a narrow lytic spectrum. Benefiting from other studies performed by our group, we fused the CD of highly soluble *E. faecalis* phage endolysins, Lys168 and Lys170 (Proença *et al*., 2012), to the CWBD of the staphylococcal insoluble endolysin Lys87 (Cantante, 2008), obtaining the chimerical endolysins Lys168-87 and Lys170-87. Remarkably, in addition to their capacity to lyse *S. aureus*, the chimeras remained active against enterococcal isolates and were extended in their lytic spectrum to coagulase-negative staphylococci and *S. pyogenes*. This extended host range suggests that the chimerical enzymes recognized common epitopes on the cell surface of these different bacterial species. The comparison of Lys168-87 and Lys170-87 lytic performances indicated that Lys168-87 was the most effective against the tested strains of *S. aureus* and of other Gram-positive species. Curiously, the chimeras seemed to follow the lytic profile displayed by the parental enterococcal endolysins when used against *E. faecalis* and *E. faecium* strains, with Lys170-87 generally producing larger and more transparent lysis halos than Lys168-87. Such result strengthens the idea that the affinity of both endolysin domains, CWBD and CD, to the PG substrate can be important for lytic activity (Low *et al*., 2011; Mayer *et al*., 2011). When Lys168-87 and Lys170-87 were used against cells resuspended in a buffer that maintained cell viability, but which was unable to support cell growth, they could lyse and kill *S. aureus*. However, when tested in rich culture media against actively growing bacteria, the chimeras failed to elicit efficient cell lysis (data not shown). This and similar observations with other lytic enzymes studied by our group led us to consider the membrane pmf, and by extension the holin function, as factors influencing endolysin activity.

It is commonly accepted that c-endolysins are synthesized in their fully active conformation, being ready to act once contact with the PG is allowed. For that reason c-endolysins exploration as enzybiotics is based on the simple idea that these enzymes should be able to effectively attack the CW of target bacteria when added from the outside. This view is now challenged by the results presented in chapter 3, as we show that healthy bacteria with fully energized CM may be able to counteract the lytic action of c-endolysins when these reach the CW from without. Cells lose this capacity under pmf-collapsing conditions, such as nutrient depletion, treatment with CM ionophores or after holin action. In fact, until now the basic concept of lysis from without had

completely disregarded that, in their natural context of action, c-endolysins always act after the holin-mediated killing of host bacteria.

The holin-mediated sensitization of bacterial cells to c-endolysins lytic action suggests that, in presence of an intact pmf, the lytic enzymes can be subjected to some sort of repression in the CW compartment. In that sense, at least some c-endolysins seem to behave similarly to e-endolysins, whose activity is known to be restrained in the CW until the holin trigger (Xu *et al*., 2004, 2005; Nascimento *et al*., 2008; Sun *et al*., 2009; Catalão *et al*., 2010; Frias *et al*., 2013). In agreement with this, we proved for the first time that the continuous Sec-mediated secretion of a c-endolysin (LysSPP1) to the CW did not interfere with cell growth, as long as the pmf was maintained. Therefore, the boosting of lytic activity upon collapse of the membrane pmf is extensible to at least some c-endolysins, irrespectively of the route followed to reach the CW (Fig. 5.1). This implies that, in addition to the two previously known roles of the holin function in canonical lysis, that is, host cell killing and endolysin transport to the CW, a third role must be added which is that of sensitizing bacteria to the lytic action of endolysins.

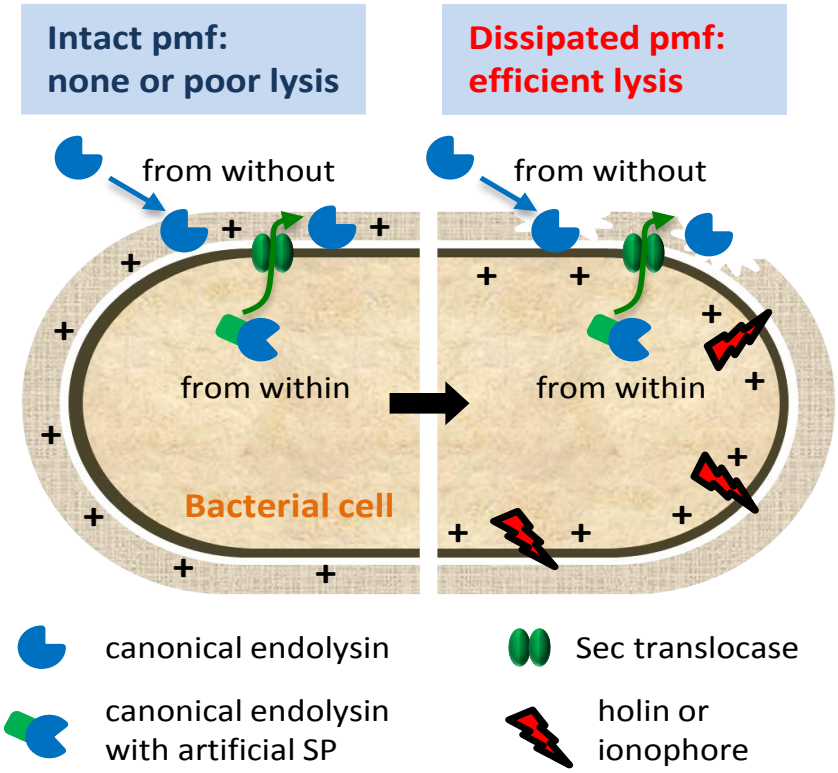

Fig. 5.1. The cellular energy is a key factor in endolysins lytic activity. Bacterial cells under conditions supporting the membrane proton motive force (pmf) can counteract the lytic activity of canonical endolysins. Pmf dissipation by the holin or by ionophores mimicking its action results in drastic increase of endolysin lytic activity. Therefore, in canonical lysis the holin action may be crucial not only for endolysin transport to the cell wall, but also to turn bacteria fully susceptible to endolysin attack.

As already mentioned, during phage infection exported and canonical endolysins always act in cells previously killed by the holin, suggesting that these proteins may have been shaped during evolution to act preferentially against dead cells. This should contribute for the fact that the amount of endolysin needed to lyse phage-infected cells is much lower than the amounts frequently used for enzybiotic therapy purposes. In the case of Gram-positive systems, this feature may provide a mechanism to avoid accidental lysis of potential new hosts if endolysins are released to the media after burst of phage-infected cells. In this line of thought, a study developed by Proença *et al.*, (2015) showed that the fusion of a specific CD of a virion-associated lysin, which is naturally designed to act outwardly in viable cells, to the CWBD of endolysin Lys170 generated an enzyme with lytic activity in rich bacterial growth media, a feature that was not observed with the parental Lys170.

The implications of our findings in the use of endolysins to eliminate bacteria *in vivo*, for instance in animal colonization or infection models, can be several. When bacteria propagate in animal hosts the growth conditions are for sure very different from those encountered in a rich culture medium, in which cells usually replicate much faster as long as nutrients are available. Nonetheless, we should considerer that when growing in animal hosts bacteria might be able to offer at least a certain level of resistance to endolysin attack. This may be one of the reasons why much higher endolysin doses are generally required to eliminate bacteria *in vivo*. In addition, the vast majority of the studies report protective rather than therapeutic endolysin effects, that is, animal survival is only achieved when endolysins are administered at the same time or soon after the deadly bacterial inoculum. In this regard, bacteria in colonizing states seem to be more prone to endolysin killing. Fortunately, the current evidences also suggest that bacteria have limits in their capacity to restrain endolysin lytic action and that this capacity varies among the different bacterium/endolysin systems and growth conditions. Therefore, we propose that the strategies aiming at the development of endolysins with therapeutic potential should contemplate the selection and/or engineering of lytic enzymes with the capacity to eliminate target bacteria in growth-promoting conditions, being these as close as possible to the ones found in *in vivo* scenarios.

As referred to above, in addition to their role in the transport of c-endolysins to the CW, the action of holins as membrane-depolarizing agents can be crucial to sensitize bacterial cells to endolysin attack. With the work presented in chapter 4 we gave the first steps to identify and characterize the holin of phage SPP1. As already observed for phages PBSX and MS6 (Catalão *et al*., 2011; Krogh *et al*., 1998), SPP1 holin function seems to require the simultaneous presence of two membrane-associated proteins, gp24.1 and gp26, since their individual expression did no interfere with cell viability. An unexpected finding was that *orf 24.1* carries an internal constitutive σ^A-dependent promoter, probably corresponding to promoter *PE5* (Alonso *et al*., 1997; Montenegro and Trautner, 1981; Stüber *et al*., 1981). Due to the presence of this promoter, we hypothesize that during SPP1 infection the endolysin LysSPP1 and the holin-like gp26 start to accumulate early and harmlessly in the host cell cytoplasm and CM, respectively. Later in infection, the other holin-like protein gp24.1 starts to be produced and inserts into the CM, probably interacting with gp26. Suddenly, when both holin-like proteins reach a critical concentration in the CM, they trigger to collapse the pmf and LysSPP1 is translocated to

the CW across the gp24.1/gp26 holes. Herein, in a dead cell, LysSPP1 is able to display full activity and quickly degrade the PG, allowing the efficient release of the newly formed SPP1 virions.

After our study a question that remains open is how pmf is involved in the regulation of endolysin activity. This is clearly a topic that deserves to be investigated as it could provide answers not only regarding endolysin regulation, but also about related phenomena such as autolysis and bacterial lysis induced by killing agents like conventional antibiotics, bacteriocins and antimicrobial peptides. A simple explanation could be that the sudden and major ion and/or pH variations in the CW environment caused by pmf collapse are directly sensed by endolysins. Another hypothesis is that the membrane energy state is sensed by TAs (and their substituents) in the CW, which could act as negative regulators of endolysins under pmf-supporting conditions. This point of view is favored by the knowledge that these polyanion polymers suffer conformational changes depending on their ionization state and have been pointed out as regulators of autolysins (Biswas *et al*., 2012). Studying the effect of mutations that change the composition and/or structure of TAs on bacterial sensitivity to endolysins could be a good starting approach.

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